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Doctoral Thesis

ROLE OF GATA4 IN PANCREATIC PHYSIOLOGY AND CARCINOGENESIS

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ABSTRACT

GATA proteins are a family of transcription factors that play a crucial role in the development of several organs and in the maintenance of their homeostasis. Specifically, Gata4 and Gata6 are known to be necessary for murine pancreas development and, recently, Gata6 has been described to be an important regulator of pancreatic acinar cell differentiation. In addition, a number of studies have attributed Gata6 a tumour suppressor role in pancreatic carcinogenesis, both in mouse and human. Yet, the role of Gata4 in pancreas homeostasis and tumour progression has not been investigated.

I have characterized Gata4 function in the murine pancreas by specifically deleting the gene during embryonic development and have found that, unlike Gata6, Gata4 is not required to maintain acinar homeostasis in the adult.

The study of Gata4 function in tumour initiation and progression by deleting it in a mutant Kras-induced model of pancreatic carcinogenesis (*Kras*;Gata4*$^{P-/-}$ mice) has revealed that Gata4-null mice develop pancreatic cancer (PDAC) in the absence of PanINs and acino-ductal metaplasia (ADM), which are believed to be the most common PDAC precursors. Lineage tracing experiments have shown that tumours can arise from adult pancreatic acinar cells. In addition, I demonstrate that Gata4 is necessary for PanIN development, as intrapancreatic delivery of IL17 in *Kras*;Gata4*$^{P-/-}$ mice, which is known to promote PanIN formation in *Kras**;*Gata4*^{WT} mice, results in the development of ductal lesions but not PanIN. I have modelled the lack of ADM/PanIN *in vitro* by establishing 3D cultures of acinar cells: treatment with EGF induces cyst formation -a surrogate for ADM- in *Gata4*^{WT} acini, but fails to completely induce such phenomenon in *Gata4*$^{-/-}$ acini. IL17 treatment induces cysts both in *Gata4*^{WT} and *Gata4*$^{-/-}$ acinar cells.

I show that Gata4 is required for the inflammatory response elicited by Kras* in the pancreas. Gata4 regulates expression of secreted factors –among them Reg3β- that activate macrophages to further enhance an inflammatory response.

I also show that *Gata4* is a *bona fide* tumour suppressor as the combined deletion of *Gata4* and *Trp53* in the mutant Kras-driven model results in acceleration of tumour progression and reduced survival.

Finally, I show that GATA4 expression is lost in a subset of pancreatic cancers in humans and that the concomitant loss of GATA4 and GATA6 is associated with very poor prognosis.

These studies support the conclusion that, in the pancreas, GATA4 plays a distinct role, different from that of GATA6.

RESUMEN

Las proteínas GATA forman una familia de factores de transcripción que juegan un papel crucial en el desarrollo de una variedad de órganos y el mantenimiento de su homeostasis. En concreto, Gata4 y Gata6 son necesarios para el desarrollo del páncreas de ratón y, recientemente, Gata6 se ha descrito como un regulador importante de la diferenciación de las células acinares pancreáticas. Además, varios estudios han atribuido a Gata6 un rol de supresor tumoral en la carcinogénesis pancreática en ratón y humano. Aun así, el rol de Gata4 en la homeostasis pancreática y la progresión tumoral no ha sido investigado.

He caracterizado el rol de Gata4 en el páncreas de ratón mediante su deleción durante el desarrollo pancreático y he observado que Gata4, contrariamente a Gata6, no es necesario para mantener la homeostasis acinar en el adulto.

El estudio de Gata4 en la iniciación y progresión tumoral mediante su deleción en un modelo de carcinogénesis inducida por Kras mutante (ratones *Kras*; Gata4*$^{P-/-}$) ha desvelado que estos ratones desarrollan cáncer de páncreas (PDAC) en ausencia de PanINs y metaplasia acino-ductal (ADM), considerados los precursores más comunes del PDAC. Experimentos de rastreo de linaje muestran que los tumores en los ratones *Kras*;Gata4*$^{P-/-}$ pueden formarse a partir de células acinares adultas. Además, he demostrado que Gata4 es necesario para el desarrollo de PanINs, ya que la administración intrapancreática en ratones *Kras*;Gata4*$^{P-/-}$ de IL17, un potente inductor de PanINs en ratones *Kras*;Gata4*^{WT}, resulta en el desarrollo de lesiones ductales, pero no de PanINs. He modelado la ausencia de ADM/PanIN *in vitro* mediante cultivos 3D de células acinares: EGF induce formación de estructuras quísticas -equivalentes a ADM- en acinos *Gata4*^{WT}, pero no logra hacerlo de igual forma en acinos *Gata4*$^{-/-}$. En cambio, IL17 induce estructuras quísticas en ambos genotipos.

He mostrado que Gata4 es necesario para la respuesta inflamatoria provocada por Kras* en el páncreas: Gata4 regula la expresión de factores secretados –como Reg3β- que activan macrófagos para inducir una mayor respuesta inflamatoria.

También muestro que *Gata4* es un gen supresor tumoral ya que su deleción junto con la de *Trp53* en el modelo de *Kras** resulta en una aceleración de la progresión tumoral y una menor supervivencia.

Finalmente, muestro que la expresión de GATA4 se pierde en un subtipo de cáncer de páncreas en humanos y que la pérdida conjunta de GATA4 y GATA6 se asocia con un peor pronóstico.

En conjunto, estos estudios respaldan la conclusión de que, en el páncreas, GATA4 juega un papel distinto al de GATA6.

TABLE OF CONTENTS

INTRODUCTION

1. THE PANCREAS

1.1. General considerations and anatomy of the pancreas

The name pancreas derives from the Greek roots 'pan' meaning 'all' and 'creas' meaning 'flesh' due to its fleshy consistency (Slack, 1982). The mature mammalian pancreas is a compound exocrine-endocrine gland that regulates essential physiologic functions such as food digestion and glucose homeostasis, respectively.

In humans, the pancreas forms a well-defined organ of 70-150 grams measuring 15-25cm in length. The terms head, neck, body and tail are used to designate regions of the organ from proximal to distal. In contrast, the shape of the pancreas in rodents is less well defined. The organ is localized in the upper region of the abdomen, between the spleen, stomach and small intestine, and it is connected to the duodenum by the ampulla of Vater, where the main pancreatic duct joins with the common bile duct (Slack, 1982) (Fig. I1A).

1.2. Histology and physiology of the pancreas

1.2.1 The exocrine pancreas

The exocrine pancreas constitutes more than 90% of the whole tissue and is composed of three distinguishable epithelial cell types: acinar, ductal, and centroacinar cells (Fig. I1B).

Acinar cells are organized into functional secretory units called acini, which are in charge of producing hydrolytic digestive enzymes at a large scale. Acinar cells have pyramidal shape with basal nuclei, regular arrays of rough endoplasmic reticulum (ER), a prominent Golgi complex and numerous secretory (zymogen) granules, containing the digestive enzymes. There are at least 22 of these, including proteases, amylases, lipases and nucleases. Most of them are secreted as inactive precursors and become activated after they enter the duodenum (Slack, 1982). The biology of acinar cells will be further detailed in a subsequent chapter.

Ductal cells form the epithelial lining of the branched tubes that deliver the enzymes produced by acinar cells into the duodenum. Ductal cells secrete mucins and fluid that flushes the acinar secretions into the intestine, as well as bicarbonate to neutralize stomach acidity. The intercalated ducts, which drain the acini, are composed of a simple epithelium of ductal cells and are surrounded by little connective tissue. As ducts become larger the epithelium becomes either cuboidal or columnar and, in the largest branches of the network, goblet cells are

intermingled with ductal cells, forming about 2% of its structure. The intercalated ducts converge to form interlobular ducts that connect the different lobes of the pancreas, merging into the main pancreatic duct, which is formed by a columnar epithelium, surrounded by abundant connective tissue (Grapin-botton, 2005; Slack, 1982).

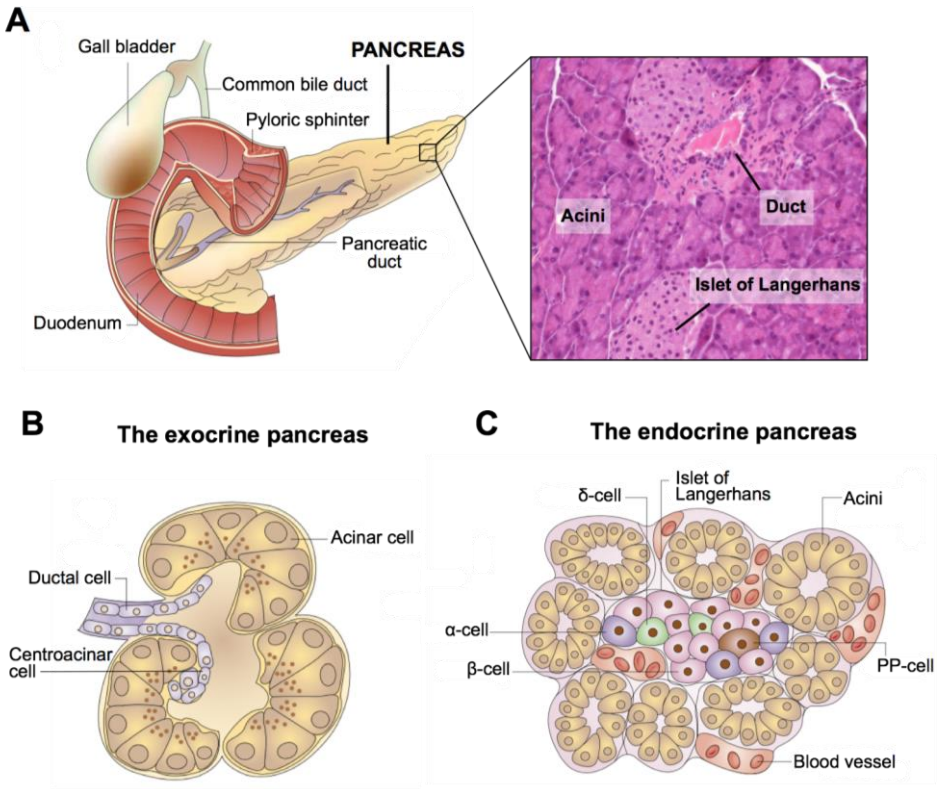

Figure I1. Anatomy of the pancreas. (A) Gross anatomy of the pancreas (left). H-E staining of the pancreas showing the exocrine (Duct and Acini) and the endocrine compartments (Islet of Langherhans). **(B)** Schematic representation of the exocrine tissue showing organization of acinar, ductal and centroacinar cells. **(C)** Schematic representation of the endocrine tissue showing an Islet of Langherhans embedded in exocrine tissue. *Adapted from Bardeesy & Depinho, 2002.*

Centroacinar cells (CACs) are located at the junction of acini and the terminal ductal epithelium, have a low cuboidal shape and are rich in mitochondria. (Slack, 1982). This cell type is poorly characterized and it is uncertain whether centroacinar and terminal duct cells represent two different cell types. They have been proposed as possible multilineage pancreatic progenitors as they rapidly proliferate upon damaging processes such as partial pancreatectomy (Hayashi *et al*., 2003); administration of streptozotocin (a chemical that is particularly toxic to the insulin-producing beta cells of the pancreas) (Nagasao, *et al*., 2003); or administration of caerulein, which induces pancreatic damage at

high doses (Gasslandek, *et al*., 1992). In addition, CACs express high levels of Aldh1, which is linked to multipotency in other systems. In the pancreas Aldh1^{high} cells have increased progenitor properties, being able to form self-renewing "pancreatospheres" in suspension and displaying spontaneous endocrine and exocrine differentiation capacity (Rovira *et al*., 2010).

1.2.1. The endocrine pancreas

The endocrine pancreas is composed by compact structures called Islets of Langerhans that are embedded in the exocrine tissue. Islets comprise 1-2% of the pancreas and have a mean diameter of 130μm in humans (Rorsman & Braun, 2013). In addition, isolated islet cells may be found dispersed in the acinar lobules or in association with ducts (MacDonald *et al*., 2010). Several hormone-producing cell types populate the islet, including β-cells (which secrete insulin), α-cells (glucagon), δ-cells (somatostatin), ε-cells (ghrelin), and PP-cells (pancreatic polypeptide). Finely tuned regulation of hormone release is achieved by coordinated interactions between the islet cells and the vascular environment, establishing hormonal homeostasis that maintain appropriate glucose levels within tissues and blood (Puri & Hebrok, 2010).

1.3. Development of the mouse pancreas

1.3.1. Morphogenesis

The pancreas originates from the primitive gut tube, which derives from the definitive endoderm, and its development is divided in three major periods: a primary transition from embryonic day (E) 9.5 to E12.5; a secondary transition from E13 to birth; and the postnatal maturation period from birth to weaning (Fig. I2).

During the primary transition, pancreas development begins with thickening of the endoderm and proliferation of early pancreatic progenitors that results in evagination of a dorsal and two smaller ventral pancreatic buds (Fig. I2-1). During bud evagination, transient epithelial stratification results in formation of microlumens, which subsequently coalesce and form continuous tubular structures (Fig. I2-2). Concomitantly, specification and patterning of early pancreatic progenitors results in formation of a bipotent stalk or “trunk” domain and multipotent “tip” domain. Cells residing at the "tip" domain are thought to include multipotent progenitor cells (MPCs) that will give rise to both endocrine and exocrine cells, whereas progeny of cells residing in the trunk will produce duct and endocrine cells (panel 3 of Fig. 1). (Benitez, *et al*., 2012).

The secondary transition represents a period of extensive epithelial expansion concomitant with differentiation of acinar, ductal and endocrine cells (Fig. I2-4). At E13, progenitors of the "tip" domain lose their multipotency and become preacinar cells, which begin to synthesize acinar digestive enzymes such as amylase, elastase, and trypsinogen. After, about E14.5, acinar cells are mainly generated by the duplication of existing acinar cells. Endocrine progenitor cells emerge from within the trunk epithelium and, following specification, they delaminate from the epithelium and coalesce to form islets. Acinar and endocrine cells go through a maturation process lasting into postnatal life (Benitez, *et al.*, 2012; Gittes *et al.*, 2009; Pan & Wright, 2011).

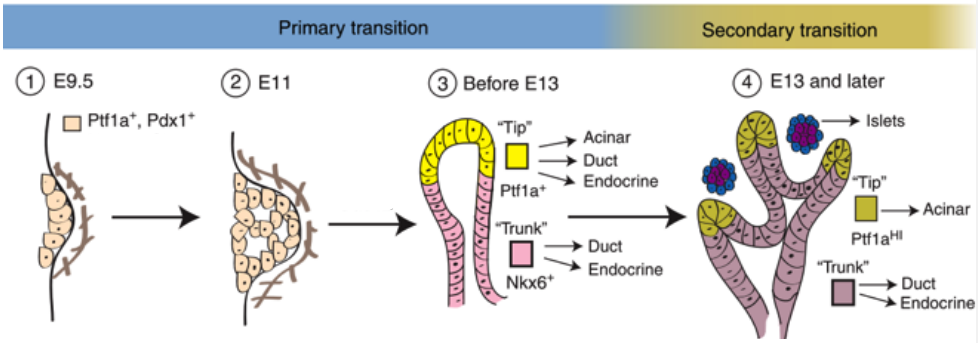

Figure I2. Pancreas development. **(1)** Pancreatic bud formation and epithelial expansion. **(2)** Microlumen formation. **(3)** Tube formation and establishment of "tip" and "trunk" domains. **(4)** Pancreatic morphogenesis, branching, cell differentiation, and isletogenesis. *Adapted from Benitez et al., 2012.*

1.3.2. Master regulators of pancreas and acinar specification

During pancreas morphogenesis, cell specification is driven by extrinsic signals from the surrounding mesenchyme and penetrating blood vessels that crosstalk with intrinsic regulation of differentiation conducted by pancreas-specific transcription programs (Magenheim *et al.*, 2011; Puri & Hebrok, 2010). The main transcription factors involved in pancreas specification are the pancreas and duodenal homeobox gene-1 (Pdx1) and the pancreatic transcription factor-1 (Ptf1a/p48) (Fig. I2-1).

Pdx1 is a homeodomain transcription factor that is expressed in mouse prepancreatic endoderm at E8.5, before the formation of pancreatic buds. Since inactivation of Pdx1 results in complete arrest of pancreas formation, it is believed to initiate pancreatic development (Offield *et al.*, 1996). In addition, lineage tracing experiments determined that Pdx1+ cells contribute to all pancreatic cell types in the mature tissue (Gu *et al.*, 2002), supporting the notion that Pdx1-expressing cells are the early precursors of all pancreatic cell types. In the adult, Pdx1 is mainly expressed in β-cells and δ-cells, and its expression in the exocrine remains low. It is required for maintaining β-cell identity and

function, and its deletion in this cell type leads to diabetes (Ahlgren *et al*., 1998; Ohlsson *et al*., 1993).

Ptf1a (or p48) is a pancreas and neural-specific class B bHLH transcription factor (Meredith *et al*., 2013). In the murine pancreas it is expressed in early pancreatic progenitors shortly after Pdx1 expression, around E9-9.5. Ptf1a is required for the evagination of the ventral buds and the growth of the dorsal bud, and it determines the pancreatic fate of Pdx1-expressing cells, as its deletion results in differentiation of progenitor cells towards an intestinal phenotype (Kawaguchi *et al*., 2002; Krapp *et al*., 1996, 1998).

1.3.3. Acinar differentiation and maturation

During primary transition, MPCs of the "tip" domain express Ptf1a that, together with Tcf12 and Rbpj, form a trimeric complex (called PTF1-J) that is required for growth and morphogenesis of pancreatic epithelium. Indeed, PTF1-J complex plays such a critical role in pancreas organogenesis that inactivation of Ptf1a or blocking its interaction with Rbpj leads to pancreas agenesis (Masui, *et al*., 2007). PTF1-J complex directly activates expression of *Ptf1a* itself (Masui *et al*., 2007) and of some digestive enzymes such as Carboxypeptidase A1 (*Cpa1*), which is considered a marker of MPCs (Q. Zhou *et al*., 2007). At the same time, cells at the "trunk" domain express Nkx6.1 and Nkx6.2 that antagonize Ptf1a, thus inhibiting MPC phenotype and directing these cells into a bipotent endocrine-ductal fate (Schaffer *et al*., 2010).

Acinar specification during the secondary transition implicates a replacement of Rbpj by Rbpjl in PTF1 complex (Beres *et al*., 2006) (FIg. I3). Rbpjl is first expressed shortly after MPCs start expressing high levels of Ptf1a (also required for acinar specification). Induction of Rbpjl transcription requires binding of the PTF1-J complex to its promoter (Masui *et al*., 2008) and, as Rbpjl accumulates during acinar cell maturation, it gradually replaces Rbpj in the complex (now termed PTF1-L).

Expression of Rbpjl as well as Ptf1a is maintained in differentiated acinar cells as they are targets of PTF1-L complex (Masui *et al*., 2008). In addition, PTF1-L complex regulates expression of 27 out of 28 major acinar secretory proteins; enzymes necessary for mitochondrial carbon, nitrogen and energy metabolism; and eight components of the intracellular protein transport apparatus. Regulation of genes involved in such processes is in accordance with acinar function: enhanced mitochondrial metabolism is necessary for the great amount of energy required for massive synthesis of secretory proteins, packaging and regulated secretion; and the proteins for intracellular transport increase the efficiency of packaging the pancreatic enzymes (MacDonald *et al*., 2010; Masui *et al*., 2010, 2007).

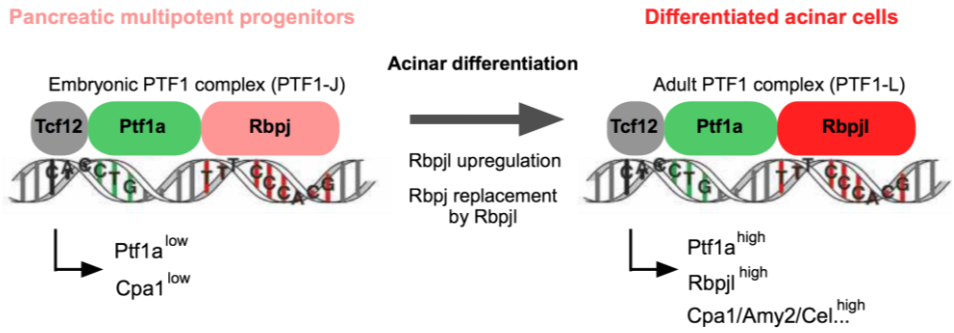

Figure I3. The PTF1 complex. In multipotent pancreatic progenitors, the embryonic form of the PTF1 complex (PFT1-J) is constituted by Ptf1a, a ubiquitous bHLH cofactor (i.e. Tcf12) and Rbpj. During development, when acinar cell specification occurs, PTF1-J binds to Rbpjl promoter. During acinar cells maturation, Rbpjl starts to accumulate, replacing Rbpj in the adult form of PTF1 complex (PTF1-L). PTF1-L promotes complete acinar differentiation, enhancing the expression of acinar specific genes, such as the digestive enzymes Caboxypeptidase A1 (*Cpa1*), Amylase 2 (*Amy2*), Carboxyl Ester Lipase (*Cel*), and others, as well as the machinery necessary for high protein synthesis and secretory functions. *Adapted from Masui et al, 2007*.

1.3.4. Maintenance of acinar differentiation in the adult murine pancreas

In addition to the PTF1 complex, other important factors regulating pancreas development and maintenance of acinar identity include Nr5a2, Gata6, Mist1 and Hnf1a.

Nr5a2 (or Lrh1) is a transcription factor of the orphan nuclear receptor family that maintains acinar differentiation, and its deletion leads to destabilisation of the mature acinar state, conversion of acinar cells to ductal-like cells, and loss of regenerative capacity following acute caerulein pancreatitis. (Flández *et al*., 2014; Holmstrom *et al*., 2011; von Figura *et al*., 2014).

Gata6 is a Zn finger transcription factor that is required for pancreas development (Carrasco *et al*., 2012; Decker, *et al*., 2006; Xuan *et al*., 2012). During post-natal maturation, Gata6 is necessary to complete acinar differentiation, to establish cell polarity and to maintain acinar cell identity in the adult. Gata6 regulates directly the promoter of genes coding for digestive enzymes and the transcription factors Rbpjl and Mist1. Pancreas-specific inactivation of Gata6 leads to massive loss of acinar cells and replacement by fat; increased acinar apoptosis and proliferation; and acinar-to-ductal metaplasia (ADM) (Martinelli *et al*., 2012). The role of Gata6 in pancreas development, homeostasis and carcinogenesis is further discussed in following chapters.

Mist1/Bhlha15 is a bHLH transcription factor that functions as a homodimer and plays an important role in maintaining acinar differentiation (Pin *et al*., 2001). Mist1 regulates apical-basal polarity, formation of gap junctions, proper

positioning of zymogen granules and their secretion; and its ablation leads to depletion of gap juntions, loss of polarity, dedifferentiation and ADM (Direnzo *et al*., 2012; Zhu *et al*., 2004).

Hnf1a (or Tcf1) is a homeobox transcription factor whose deletion results in reduced expression of Ptf1a and digestive enzymes. Hnf1a-null acini show suboptimal secretory responses to cerulein. *In silico* and ChIP-PCR studies revealed that Hnf1a binds to, and regulates, the promoter of Nr5a2 (Molero *et al*., 2012).

1.4. Pancreatic acinar cell biology

1.4.1. Acinar cell function in food digestion

Pancreatic acinar cells produce the majority of the digestive enzymes that are in charge of digesting food in the intestine for further absorption. Acinar cells have the highest rate of protein synthesis of all mammalian cell types in order to generate the different enzymes involved in the hydrolysis of proteins (trypsin, chymotrypsin, carboxypeptidase, gelatinase and elastase), lipids (lipases), polysaccharides (amylases), and nucleic acids (deoxyribonucleases and ribonucleases) (Case, 1978). This massive protein production is achieved due to a very high rate of transcription of genes coding for digestive enzymes, a very abundant rough ER and an efficient system for storage and secretion.

Transcripts coding for digestive enzymes constitute around 80% of all mRNAs of acinar cells (MacDonald *et al*., 2010). Upon translation, immature proteins are modified in the lumen of the ER and in the Golgi complex, and finally, undergo concentration and packaging into highly specialized storage structures called zymogen granules (Farquhar & Palade, 1998). In addition to protein synthesis, the ER also plays an important role in zymogen granule secretion. Upon neurohumoral stimulation of acinar cells receptors, the Ca^{2+} ions stored in the ER flux into the cytosol, resulting in secretion of zymogen granules into the lumen of the ducts (Petersen & Tepikin, 2008).

Among all digestive enzymes produced by acinar cells, proteases are synthesized as inactive pro-enzymes that are activated in the duodenum through a cascade of enzymatic reactions. First, in the duodenal lumen, enteropeptidase activates trypsinogen yielding trypsin, the active enzyme. Trypsin further catalyses the activation of other pro-enzymes (including remaining trypsinogen), including procarboxypeptidase (to carboxypeptidase), chymotrypsynogen (to chymotripsin), proelastase (to elastase), and others (Whitcomb & Lowe, 2007). Trypsin also undergoes self-hydrolysis providing a negative regulatory loop to the digestive process (Whitcomb *et al*., 1996).

1.4.2. Acinar cell plasticity

Adult pancreatic acinar cells display a wide plasticity and are able to give rise to other differentiated cell types, both pancreatic and non-pancreatic.

The best studied process is the ability of acinar cells to transdifferentiate to ductal-like cells in a process known as acinar-to-ductal metaplasia (ADM) (Fig. I4), which was first observed *in vitro* (Hall *et al*., 1992; Vila, *et al*., 1994) and later shown *in vivo* to be a true transdifferentiation event through lineage tracing experiments (Means *et al*., 2005). ADM takes place in the damaged pancreas and it plays a crucial role in tissue regeneration. The signalling pathways triggering ADM, the process itself, and its consequences will be detailed later in this chapter.

Prior to the establishment of ductal structures during ADM, acinar cells undergo a process of dedifferentiation (Fig. I4), in which genes that are hallmark of complete differentiation are downregulated (i.e. Ptf1a, digestive enzymes). Acinar dedifferentiation occurs in response to pancreatic injury, upon loss of cell–cell and cell–matrix contacts, loss of polarity, Kras hyperactivity and increased inflammatory signalling. Under these circumstances dedifferentiated acinar cells transdifferentiate to a duct-like phenotype and complete the ADM process required for pancreatic regeneration (Storz, 2017). In addition, acinar cell dedifferentiation is also observed in human cases of pancreatitis and in mouse models of acute and chronic pancreatitis, as well as in murine acinar cells cultured in suspension (Pinho *et al*., 2011).

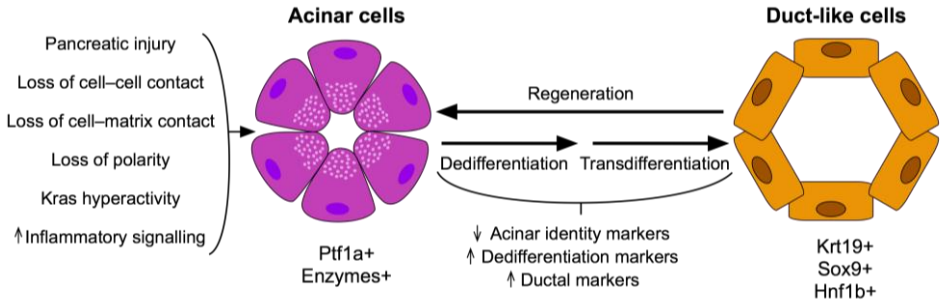

Figure I4. Acinar-to-ductal metaplasia. Pancreatic injury, loss of cell–cell and cell–matrix contacts, loss of polarity, Kras hyperactivity and increased inflammatory signalling can drive acinar cells to undergo dedifferentiation (loosing expression of differentiation markers such as Ptf1a or digestive enzymes) and transdifferentiation to a duct-like phenotype (expressing Krt19, Sox9 and Hnf1b), which is needed for pancreatic regeneration. *Adapted from Storz, 2017.*

Acinar cells also have the ability to differentiate to endocrine cells. This process has been described to happen *in vitro* (Baeyens *et al*., 2009) and, most importantly, *in vivo* generation of β-cells from acinar cells has been achieved in mice by re-expressing the developmental transcription factors Ngn3, Pdx1 and

Mafa (Zhou *et al*., 2008). Although the process in mice needs to be completely optimized (Akinci *et al*., 2012), there is evidence that also human acinar cells can convert to β-like cells (Lemper *et al*., 2014). Lately, it has also been demonstrated that acinar cells are able to acquire a phenotype similar to to α- and δ-cells (W. Li *et al*., 2014).

Acinar cells can also transdifferentiate into hepatocytes, which share a common endodermal origin with pancreatic cells. It has been shown that foci of hepatic cells appear in the pancreas of rats exposed to a low-copper diet (Dabeva *et al*., 1995) and in rats treated with ciprofibrate, a peroxisome proliferator (Reddy *et al*., 1984). Transdifferentiation into hepatocytes has been shown to happen *in vitro* when culturing exocrine cells in the presence of the synthetic glucocorticoid dexamethasone (Shen *et al*., 2000; Shen *et al*., 2003).

Transdifferentiation of acinar cells into ductal, endocrine or hepatic cells are cases of epithelial-to-epithelial transition. However, acinar cells can also lose their epithelial characteristics and transdifferentiate into adipocytes, thus undergoing an epithelial-to-mesenchymal transition (EMT). This process has been reported to occur upon c-Myc inativation (Bonal *et al*., 2009) or, as already mentioned, upon *Gata6* deletion in mouse pancreas (Martinelli *et al*., 2012). This event is relevant because it may be occurring in ageing and pancreatitis, conditions that are characterized by exocrine pancreas involution and replacement with adipose tissue (Bonal *et al*., 2009).

1.4.2.1. Acinar-to-ductal metaplasia (ADM)

Metaplasia is a broad histological term that is defined by the conversion or replacement of one differentiated cell type with another (Slack & Tosh, 2001). In the pancreas, the most relevant metaplastic process is the conversion of acinar cells to ductal through loss of acinar genes (dedifferentiation) and induction of genes typically expressed by ductal cells, such as the intermediate filament Krt19 (Bouwens, 1998) or the transcription factors Sox9 and Hnf1b (Reichert & Rustgi, 2011) (Fig. I4).

While in the normal pancreas the predominant cells are acinar, in the diseased pancreas acinar tissue is often replaced by metaplastic ductal lesions (MDL), which are the result of ADM. MDL can be observed in cases of pancreatitis as well as in pancreatic cancer (Strobel *et al*., 2007). Depending on the morphological features of MDL it is possible to distinguish two types of lesions: (I) tubular complexes (TC) and (II) mucinous metaplastic lesions, recently agreed to be termed Pancreatic Intraepithelial Neoplasia (PanIN), and considered a precursor of pancreatic cancer (Hruban *et al*., 2001).

TC are defined as cylindrical tubes with an often wide lumen lined by a

monolayer of flattened duct-like cells (Lechene de la Porte *et al*., 1991; Willemer *et al*., 1989). In humans, TC have been observed in pancreatitis, both acute (Willemer & Adler, 1989) and chronic (Bockman *et al*., 1982). In rodents, TC have been also identified in models of pancreatitis (Lechene de la Porte *et al*., 1991), in models of pancreatic cancer induced by carcinogens (Bockman *et al*., 2003), upon 90% pancreatectomy in a model for pancreatic regeneration (Tokoro *et al*., 2003), and in genetically engineered mouse models (GEMMs) of pancreatic carcinogenesis (Kong *et al*., 2016; Wagner *et al*., 1998).

PanINs are defined as microscopic papillary or flat non-invasive neoplasms, characterized by columnar to cuboidal cells with varying amounts of mucin and increasing degrees of cytological and architectural atypia. They were initially thought to arise in the pancreatic duct, but recent studies have attributed them an acinar origin (Buchholz *et al*., 2005; Guerra *et al*., 2007; Hruban *et al*., 2001). Development of PanIN lesions and their role as putative precursor lesions of pancreatic cancer will be discussed in following chapters.

1.4.2.2. Signalling leading to ADM

It is believed that the proliferative duct-like structures resulting from ADM are able to regenerate the acinar cell mass that has been lost during damaging processes of the pancreas such as pancreatitis (Desai *et al*., 2007; Fendrich *et al*., 2008; Strobel *et al*., 2007). Upon pancreatic damage/stress, a number of transcription factors and signalling pathways that result in ADM are activated in order to promote an inflammatory response and subsequent tissue repair.

Mitogen activated protein kinase (MAPK) pathway has been widely implicated in the development of ADM at the ligand, receptor and signalling pathway level. Overexpression of ligands that activate epidermal growth factor receptor (EGFR), which subsequently activates MAPK pathway, results in acinar metaplasia (Means *et al*., 2003; Sandgren *et al*., 1990). EGFR itself has also been described to be necessary for the development of acinar metaplasia, even when the downstream MAPK pathway is manipulated to be constitutively activated by mutant Kras (Ardito *et al*., 2012; Navas *et al*., 2012). Inhibition of the MAPK signalling transducer MEK has also attributed to this kinase a crucial role in ADM (Halbrook *et al*., 2017).

Notch pathway has also been involved in ADM. Actually, activation of EGFR by TGF-α results in upregulation of the Notch signalling pathway (Miyamoto *et al*., 2003), linking MAPK and Notch signalling in metaplastic lesions. In addition, the metalloproteinase MMP-7 has been found to be necessary and sufficient to induce ADM through activation of Notch signalling (Sawey *et al*., 2007).

The activation of **nuclear factor kappa B (NF-κB)** has also been determined to

play an important role in ADM. NF-κβ is a key factor in the development of inflammatory responses (Tak & Firestein, 2001), a process tightly linked to ADM. It has been shown that, upon pancreatic damage, macrophages infiltrate the pancreas and secrete inflammatory cytokines, such as Ccl5 and Tumour Necrosis Factor (TNF), which drive ADM through activation of NF-κB (Liou *et al*., 2013). In addition, high activity of NF-κB has been linked to a higher incidence of metaplastic lesions and an overall increase in pancreatitis severity (Huang, H. *et al*., 2013).

As said, the metaplastic process converting an acinar cell to a ductal one requires expression of different genes, which is achieved by activation of a subset of transcription factors. Under inflammatory conditions or upon oncogene activation (both promoting ADM) Sox9 expression in acinar cells increases and stimulates transcription of genes that lead to ADM. According with these findings, SOX9 expression in patient tumour samples is elevated at all stages of preneoplastic lesions and in pancreatic cancer, and positevily correlates with increased activation of EGFR pathway. Pdx1 is upregulated during pancreatitis, in all types of tumour precursor lesions (including PanIN and other neoplasms), as well as in pancreatic tumours. Pdx1 regulates ADM through activation of Signal Transducer and Activator of Transcription 3 (STAT3), which is also a regulator of inflammation. Other factors required for ADM include Hepatocyte Nuclear Factor 6 (Hnf6), which represses acinar genes and upregulates ductal genes in both mouse and human acinar cells; and cMyc and Klf4, which are required to initiate the ADM process in mice (reviewed in Storz, 2017).

2. DISEASES OF THE EXOCRINE PANCREAS

The major exocrine pancreas diseases include pancreatitis (acute and chronic) and pancreatic cancer; and acinar cells play a central role in the pathogenesis of both conditions. In pancreatitis, injury is initiated in acinar cells probably as a consequence of the premature intracellular activation of digestive enzymes (Lerch *et al*., 2000). Repeated attacks of acute pancreatitis have the potential to evolve into a chronic disease characterized by loss of pancreatic function and fibrosis (Vonlaufen *et al*., 2008); and chronic pancreatitis is a major risk factor for cancer, specially when caused by genetic factors (Raimondi *et al*., 2010). Pancreatic cancer has historically been thought to arise from ductal epithelium, yet murine models have revealed that acinar cells are capable of transforming into PDAC through a process involving oncogene mutation, deletion of tumour suppressors, and activation of developmental pathways (Reichert *et al*., 2016), highlighting the importance of these cells in all forms of panceatic disease.

2.1. Pancreatitis

2.1.1. General considerations and aetiology

Pancreatitis is a necroinflammatory condition of the pancreas that can present with acute and/or chronic manifestations. Acute pancreatitis (AP) is characterized histologically by oedema, acinar cell vacuolization/necrosis and parenchymal inflammatory infiltrates (Fig. I5A). Although AP is usually a mild and self-limiting process, 10–15% of cases are severe and associate with multi-organ dysfunction and high mortality (Vonlaufen *et al*., 2008). Chronic pancreatitis (CP) is characterized by acinar atrophy, fibrosis (Fig. I5B), and loss of endocrine and exocrine function. It is believed to occur as a result of repeated episodes of AP and in approximately 50% of cases is associated with alcohol abuse (Whitcomb, 2012).

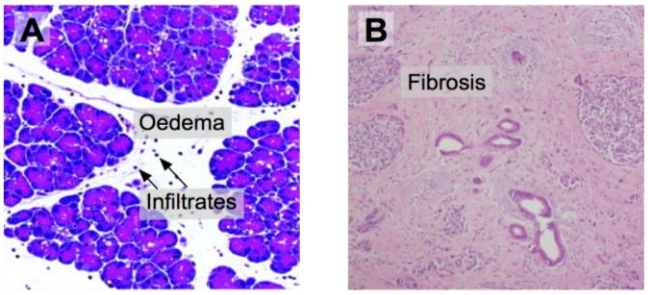

Fig I5. Pancreatitis. (A) High-magnification histology of caerulein-induced pancreatitis in mouse pancreas, showing oedema and immune infiltrates. **(B)** Low-magnification image of chronic pancreatitis in human, showing the prominent fibrotic reaction.

Regardless of its aaetiology, pancreatitis is thought to be initiated by premature activation of digestive enzymes within the acinar cell leading to autodigestion of the gland in the most severe cases. The most common causes of AP are alcohol abuse or occlusion of the pancreatic duct by gallstones. Drugs, viruses, venoms, hypercalcemia and hyperlipidemia represent rare causes. Some forms of pancreatitis that were initially classified with unknown aetiology (idiopathic pancreatitis) are now known to be caused by inherited germline mutations. These include activating mutations in the trypsinogen gene *PRSS1,* which enhance protein auto-activation and result in proteolysis and activation of other digestive enzymes within acinar cells; and inactivating mutations of genes encoding proteins that inhibit protease activity, such as *SPINK1,* or chymotrypsinogen C (*CTRC*) that degrades trypsinogen and trypsin with high specificity. Other genetic causes of pancreatitis include mutations in the cystic fibrosis transmembrane conductance regular gene (*CFTR*) and the calcium sensing receptor gene (*CASR*), among others (Whitcomb, 2012). Autoimmune and tropical pancreatitis represent less common causes, usually presented as chronic manifestations from the beginning (Vonlaufen *et al*., 2008).

2.1.2. Molecular and cellular events during pancreatitis

Acute pancreatitis results from altered physiological stimuli and cellular responses. In normal conditions, both neural (acetylcholine) and humoral (cholecystokinin, CCK) pathways stimulate acinar cells to respond to meal, inducing digestive enzyme secretion. However, abnormal signalling consisting of supraphysiologic concentrations of CCK can initiate pancreatitis. Although there is no evidence that levels of CCK necessary to cause pancreatitis (10-fold greater than in response to meal) are reached in pathological settings, this concept has been widely used to experimentally promote pancreatitis in rodents by administrating supramaximal doses of the CCK analog caerulein. (Gorelick & Thrower, 2009).

During the initial phases of AP, zymogens are activated within acinar cells, a process that is mediated by cathepsin B upon fusion of zymogen granules and lysosomes (Halangk *et al*., 2000), which leads to trypsin release and subsequent proteolysis and activation of other pro-enzymes. There are two mechanisms protecting from intracellular activation of trypsinogen: the presence of a trypsin inhibitor (SPINK1) that binds trypsin and blocks its activity, and the presence of proteases that degrade trypsin. As commented before, mutations in genes coding for these proteins predispose to risk of developing pancreatitis (Gorelick & Thrower, 2009)

Acute changes in cytosolic Ca^{2+} signalling are also associated with several forms of AP. Pathological Ca^{2+} signalling (stimulated mainly by bile and alcohol metabolites) is associated to trypsin activation, vacuolization and necrosis.

Causes of such cell damage include increased release of Ca^{2+} from intracellular ER stores, and/or increased Ca^{2+} entry through the plasma membrane (J. Li *et al*., 2014).

In the early stages of pancreatitis, acinar cells produce cytokines such as TNFa, IL-6, IL-10 and the chemokine monocyte chemoattractant protein-1 (MCP-1), suggesting that the initial inflammatory responses and signals that recruit leukocytes can originate in injured acinar cells (Habtezion, 2015). Neutrophils play a pathogenic role in the early phase of AP as they are the first cells recruited to the injury site and contribute to trypsinogen activation and progression to severe pancreatitis through generation of NADPH oxidase products (e.g. hydrogen peroxide and superoxide). These reactive oxygen species (ROS) oxidize proteins, enhancing their susceptibility to hydroliysis by serine proteases (Gukovskaya *et al*., 2002). Rapid neutrophil infiltration is followed by macrophage recruitment, playing both cell populations a key role in inflammation and repair. However, when this balance goes awry, permanent inflammatory responses can lead to recruitment of proinflammatory macrophages that impair regeneration and re-differentiation of acinar cells. In addition, continuous episodes of AP (chronic pancreatitis) lead to acinar atrophy and fibrosis, the formation of which is mediated by pancreatic stellate cells (PSC) that are scattered in the pancreatic parenchyma. In response to chronic injury, PSC become activated, acquire myofibroblast features, proliferate and secrete extracellular matrix components and growth factors. (Apte & Wilson, 2003; Habtezion, 2015).

2.1.3. Regeneration of the pancreas after pancreatitis: role of ADM

Destruction of large amounts of a specific cell type may be compensated by proliferation of residual surviving cells, or by neogenesis from a stem cell compartment. In the pancreas, regeneration caused by proliferation of surviving cells was first demonstrated for endocrine β-cells (Dor *et al*., 2004) and was later extended to acinar cells in models of damage such as caerulein pancreatitis and partial pancreatectomy (Desai *et al*., 2007; Fendrich *et al*., 2008; Strobel *et al*., 2007). However, it is not clear how replacement of acinar tissue occurs: some reports argue that proliferation of surviving differentiated acinar cells predominates and that metaplastic lesions have a minor role (Desai *et al*., 2007; Strobel *et al*., 2007); while others claim that mature exocrine cells regenerate pancreatic epithelium through formation of metaplastic ductal intermediates through activation of Hedgehog (Fendrich *et al*., 2008), Notch (Siveke *et al*., 2008) and Wnt signalling (Morris *et al*.,2010).

It is believed that loss of acinar identity (dedifferentiation) and reactivation of developmental signalling pathways might provide protective mechanisms by which the acinar cells can undergo regeneration and repair. Since acinar cells

produce enormous amounts of digestive enzymes, reduced protein synthesis might provide cells with the opportunity to repair injury-related damage (Stanger & Hebrok, 2013). While transient metaplasia resulting from activation of developmental pathways allows proper cell regeneration, it has been demonstrated that mutations in the Kras oncogene promote permanent ADM and establishment of putative cancerous lesions (PanINs) by blocking acinar regeneration. Upon acute pancreatitis in wild-type mice, the Wnt target β-catenin is activated (through protein stabilization) allowing acinar regeneration. However, β-catenin stabilization does not occur upon pancreatitis in cells carrying Kras mutations, therefore blocking acinar regeneration and resulting in the formation of permanent metaplastic lesions. Interestingly, forcing β-catenin signalling antagonizes the ability of Kras to reprogram acini into preneoplastic precursors (Morris *et al*., 2010).

2.1.4. Pancreatitis as a major risk factor for pancreatic cancer development

Chronic pancreatitis is a major risk for pancreatic cancer development in humans. A meta-analysis including 22 studies demonstrated a relative risk of 13.3 for developing pancreatic cancer in those patients with chronic pancreatitis, with a 10-20 year lag between the incidences of pancreatitis and malignancy. In addition, it was found that the relative risk for PDAC development in cases of hereditary chronic pancreatitis raised to 69.0 (Raimondi *et al*., 2010). Likewise, chronic pancreatitis induced by repeated injections of caerulein has been broadly used as a model to accelerate carcinogenesis in mice (Guerra *et al*., 2007, 2011). Similarly, a single episode of acute pancreatitis increases the incidence of neoplastic lesions and pancreatic cancer in models of Kras-induced carcinogenesis (Carrière *et al*., 2009; 2011; Flandez *et al*., 2014).

2.2. Pancreatic cancer

2.2.1. Incidence and mortality

Pancreatic cancer (or Pancreatic Ductal Adenocarcinoma, PDAC) is a highly lethal disease, for which mortality rates are similar to incidence, and a 5-year survival as low as 6% in the USA. The poor survival can be attributed to several factors, the major problem being the late stage at which patients are diagnosed. The majority of patients with PDAC are asymptomatic until the disease is in an advanced stage. Only 20% of patients are eligible for surgery, and even after potentially curative resection, most will eventually recur, the 5-year survival being only 25%. Other factors that worsen survival include high incidence of metastasis, with about 90% of cases presenting distant metastasis, as well as the

fact that pancreatic tumours are highly resistant to chemotherapy and radiotherapy (Kamisawa *et al*., 2016). In addition, PDAC is generally diagnosed at an advanced age and patients often present with cachexia. For 2017, the American Cancer Society estimates that 53,670 people will be diagnosed with pancreatic cancer in the USA and 43,090 will die of the disease (R. L. Siegel, Miller, & Jemal, 2017).

2.2.2. Risk factors

The risk factors associated with the development of pancreatic cancer are classified into modifiable -related to environment and lifestyle- and genetic (reviewed in Becker *et al*., 2014), and are listed below:

2.2.2.1 Modifiable risk factors

Smoking is the most important modifiable risk factor for PDAC, contributing to 20-35% of the cases. A pooled analysis performed in 2012 found the risk of cigarette use to be 2.2-fold and, even after 10 years of smoking cessation, a modestly elevated risk of 1.48 remains, which disappears after 20 years of cessation (Bosetti *et al*., 2012). It is likely that PDAC develops from exposure to tobacco-related carcinogens such as nitrosamines and polycyclic aromatic hydrocarbons, as well as their metabolites, which reach the pancreas through circulating blood and cause mutations in both protoncogenes and tumour suppressors. However, recent studies have shown that additional effects from other tobacco smoke components such as nicotine can also contribute (Hermann *et al*., 2014).

Alcohol ingestion has been found to be associated with PDAC, but the current evidence indicates that only heavy alcohol usage (three or more drinks per day) associates with 1.22 to 1.36-fold increase risk of developing PDAC, with a dose response relationship (Genkinger *et al*., 2009; Tramacere *et al*., 2010). High alcohol consumption contributes to pancreatitis, which may be an intermediate mechanism contributing to the increased risk of PDAC. In addition, carcinogens such as acetaldehyde (an ethanol metabolite) can contribute directly to carcinogenesis (Duell, 2012).

As discussed previously, **chronic pancreatitis** is a major factor for developing pancreatic cancer with a 13.3-fold increase risk. However, only 5% of patients with PDAC have a medical history of chronic pancreatitis (Raimondi *et al*., 2010).

Diet and obesity can also increase the risk of developing PDAC. Specifically, diet including red meat (1.29 risk) and processed meat (1.19 risk) (Larsson *et al*., 2012); or increase in body mass index (relative risk of 1.12 for each 5kg/m^2) (Larsson *et al*., 2007) are thought to induce pancreatic hypertrophy and

hyperplasia in response to abundant cholecystokinin secretion, which may favour the carcinogenic process (Jarosz *et al*., 2012).

Meta-analysis have demonstrated an association between **diabetes mellitus**, both type 1 and 2, and pancreatic cancer, with odds ratios of approximately 2.0 and 1.8, respectively (Huxley *et al*., 2005; Stevens *et al*., 2007). There is much debate about the molecular mechanisms accounting for such an association as well as the possible confounding by the presence of the tumour. Insulin is growth promoting and, thus, chronic insulinemia may result in increased cellular proliferation and decreased apoptosis, a mechanism that might contribute to PDAC. Additionally, the oxidative stress from hyperglycemia may be the cause of cell damage that could contribute to the development of neoplasm (Larsson *et al*., 2007; Stocks *et al*., 2009; Stolzenberg-Solomon *et al*., 2005).

Other factors that have been associated with the risk for developing pancreatic cancer include have undergone **surgeries** such as cholecystectomy (1.23, risk) (Lin *et al*., 2012) or gastrectomy (1.54 risk) (Y. Gong *et al*., 2012); and **infections** with H. Pylori (1.38 risk) (Raderer *et al*., 1998). There is also some evidence for a link between hepatitis B and pancreatic cancer (Hassan *et al*., 2008), as well as hepatitis C (El-serag *et al*., 2010). Exposure to **hydrocarbons** such as chlorinated hydrocarbons and polycyclic aromatic hydrocarbons positively correlate with increased risk to develop PDAC, yet consistently statistically significant results have not been found with either of these two occupational exposures (Andreotti *et al*., 2012).

Concentrations of trace elements (metals) can also affect the risk of developing PDAC. Interestingly, it has been reported that individuals with high concentrations of cadmium, arsenic and lead have an increased risk of PDAC (3.58, 2.02, and 6.26-fold increased risk, respectively), while high levels of selenium and nickel are inversely associated with risk of PDAC (0.05 and 0.27, respectively) (Amaral *et al*., 2013).

Although the majority of studies have reported conditions that increase PDAC risk, it has also been found that asthma and nasal allergies have an opposite effect and protect against PDAC development. Concretely, asthma is associated with lower risk of PDAC (OR 0.64), and the effect is even greater in long-standing asthma (>=17 years) (OR 0.39). Similarly, nasal allergies and related symptoms are associated with lower risk of PDAC (OR 0.66) (Gomez-rubio *et al*., 2015).

2.2.2.2 Genetic risk factors

In regard to genetic susceptibility, PDAC can be classified as hereditary or sporadic. In the first group, there is a strong inherited predisposition to pancreatic cancer in three distinct clinical settings: hereditary tumour

predisposition syndromes, syndromes associated with chronic inflammation of the pancreas and familial pancreatic cancer (FPC). In the second group, although environmental factors are the main drivers of pancreatic carcinogenesis, the presence of certain genetic variants (SNPs) has also been shown to modulate the risk of developing PDAC.

Hereditary pancreatic cancer

The first setting of hereditary pancreatic cancer comprises familial cancer syndromes known to be associated with an increased risk of pancreatic cancer, including Peutz–Jeghers syndrome, familial atypical multiple mole melanoma (FAMMM), hereditary breast–ovarian cancer (HBOC), hereditary non-polyposis colorectal carcinoma (HNPCC or Lynch syndrome), and familial adenomatous polyposis (FAP). It was estimated that these cancer syndromes account for 15–20% of hereditary pancreatic cancer cases (Hruban *et al*., 2010). The genes typically mutated in each syndrome as well as the relative risk (RR) of developing PDAC are summarized in Table I1.

Syndrome	Genes affected	Risk	References
Peutz-Jeghers	*STK11*	132	Giardiello *et al*., 1987; Jenne *et al*., 1998
FAMMM	*p16INK4A*	13-22	Lynch *et al*., 2008; Vasen *et al*., 2000
HBOC	*BRCA1, BRCA2, PALB2*	3.5-10	Consortium, 1999; Lynch *et al*., 2005; Murphy *et al*., 2002
HNPCC	*MLH1, MSH2, MSH6, PMS2, EPCAM*	8.6	Kastrinos *et al*., 2009
FAP	*APC*	4.5-6	Giardiello *et al*., 1993
Ataxia telangiectasia	*ATM*	2	Morrell *et al*., 1990; Swift *et al*., 1987

Table I1. **Familial cancer syndromes associated with increased risk of PDAC development**. Risk column expresses the relative risk of developing PDAC expressed as fold increase over general population. Abbreviations: FAMM, familial atypical multiple mole melanoma; HBOC, hereditary breast-ovarian cancer; HNPCC, hereditary non-polyposis colorectal carcinoma; FAP, familial adenomatous polyposis.

The second setting for an inherited predisposition to pancreatic cancer includes hereditary pancreatitis and cystic fibrosis. As described earlier, hereditary pancreatitis is caused by mutations in genes that result in premature and persistent activation, or reduced deactivation of pancreatic proteases, leading to pancreatic injury (*PRSS1, SPINK1*). A 2010 meta-analysis found a relative risk of 69 for PDAC in patients with hereditary pancreatitis compared to the general

population (Raimondi *et al*., 2010). Cystic fibrosis is caused by homozygous mutations in *CFTR* gene, and predisposes with 5.3-fold greater risk of PDAC development (Maisonneuve *et al*., 2007). However, *CFTR* mutations inherited in a heterozygous fashion also confer a 4-fold greater chance of developing chronic pancreatitis, and therefore, increase risk for PDAC development (Weiss *et al*., 2005)

The third setting is familial pancreatic cancer (FPC) syndrome, which accounts for the majority of hereditary pancreatic cancer, around 80% of cases (Hruban *et al*., 2010). FPC is defined as having 2 or more first-degree relatives (FDRs) with PDAC with no known genetic cause, and accounts for 4-10% of all PDAC cases. It has been described a nine-fold greater risk of developing PDAC among individuals with an FDR with PDAC in the setting of FPC, compared to a 1.8-fold greater risk for those with an FDR with sporadic PDAC (Klein *et al*., 2004). Additionally, among FPC kindreds, having two or three FDRs with PDAC was associated with a 6.4-fold and 32-fold greater risk of developing PDAC, respectively (Klein *et al*., 2004). FPC has several epidemiological features that distinguish it from sporadic PDAC. Similarly to other familial cancers, FPC shows a trend towards a younger onset (FPC: age 58-68; compared to sporadic PDAC: age 61-74). Similar to the sporadic cases, smoking and diabetes also increase the risk for FPC (Matsubayashi *et al*., 2017). Although linkage analysis have determined that mutations in *PALLD, BRCA2, PALB2, ATM* are the cause of FPC in some families, the genetic basis of FPC still remains to be established in the majority of cases (Bartsch *et al*., 2012).

Sporadic pancreatic cancer

Genetic predisposition to sporadic pancreatic cancer has been assessed through genome wide association studies (GWAS). These studies have provided with relevant information on allelic variants that predispose to or protect from PDAC development, and the gene to which the variant is associated. Some of these genes are listed in Table I2.

2.2.3. Histopathology of PDAC

Among all the different cancer types that can arise from the exocrine pancreas, Pancreatic Ductal Adenocarcinoma (PDAC) is the most common pancreatic neoplasm and accounts for >85% of pancreatic tumour cases (Hezel *et al*., 2006). It receives this name as a result of the histological resemblance to normal ductal cells. Although other uncommon tumours of the exocrine pancreas have been described, including Signet-ring cell carcinoma, undifferentiated anaplastic carcinoma, mixed ductal-endocrine carcinoma, osteoclast-like giant cell tumour, serous cystadenocarcinoma, mucinous cystadenocarcinoma, acinar cell

carcinoma or pancreatoblastoma, among others (reviewed in Klöppel *et al*., 1996), pancreatic cancer and PDAC are used as synonyms in the present work.

Gene identified	Study	Gene identified	Study
CEL	Dalva *et al*., 2017	*NR5A2*	Petersen *et al*., 2010
LINC-PINT	Wolpin *et al*., 2014	*CLPTM1L- TERT*	Petersen *et al*., 2010
BCAR1/CTRB1/CTRB2	Wolpin *et al*., 2014	*LINC00673*	Childs *et al*., 2015
PDX1	Wolpin *et al*., 2014	*SUGCT*	Childs *et al*., 2015
ZNRF3	Wolpin *et al*., 2014	*TP63*	Childs *et al*., 2015
TERT	Wolpin *et al*., 2014	*ETAA1*	Childs *et al*., 2015
PVT1	Wolpin *et al*., 2014	*ABO*	Amundadottir *et al*., 2009
BRCA2	L. Huang *et al*., 2013	*TERT*	Bao *et al*., 2017
MAP2K4	L. Huang *et al*., 2013		

Table I2. Genes presenting genetic variants that modulate predisposition to PDAC development.

Among all the low-penetrance variants that influence predisposition to PDAC development, the ones affecting the AB0 system are well defined. Non-0 blood groups have been associated with a 1.3 higher risk of PDAC (Amundadottir *et al*., 2009; Egawa *et al*., 2013; Risch *et al*., 2010). In addition, a meta-analysis found that having an O blood group was associated with a relative risk of 0.79 for the development of PDAC, indicating that this variant is protective (Amundadottir *et al*., 2009; Iodice *et al*., 2010).

At the macroscopic level, PDAC usually consists of a firm, highly sclerotic mass, with poorly defined edges and long tongues of carcinoma extending beyond the main tumour (Fig. I6A). Microscopically, pancreatic cancer is composed of an infiltrating gland-forming neoplastic epithelium displaying variable differentiation features, ranging from a glandular appearance (differentiated) to a mesenchymal (less differentiated) aspect (Maitra & Hruban, 2008). However, the majority of tumours are reported by pathologists as being "moderately differentiated". Neoplastic cells upregulate the expression of mucins (especially MUC1 and MUC4), a feature that has contributed to establish a progression model to PDAC from non-invasive lesions that also produce mucins (Balagué *et al*., 1994; Jonckheere *et al*., 2010). A hallmark of PDAC is that epithelial compartment of the tumour is surrounded with a dense stroma of fibroblasts and inflammatory cells, called desmoplastic reaction, which can comprise as

much as 90% of the total tumour (Fig. I6B, C). Pancreatic cancers are extremely infiltrative neoplasms: vascular and perineural invasion are present in the majority of surgically resected cancers, and metastases to regional lymph nodes, the liver, and distant sites are also common (Maitra & Hruban, 2008).

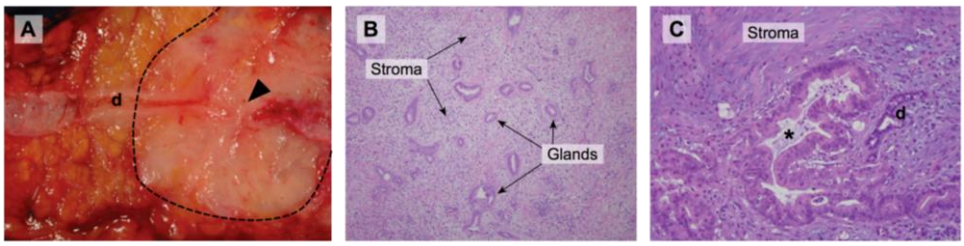

Figure I6. Pathology of PDAC. (A) Gross photograph of an infiltrating adenocarcinoma. Note the dramatic narrowing (arrowhead) of the pancreatic duct (d) associated with the poorly defined white neoplasm (dashed line). **(B)** Low-magnification image of a differentiated infiltrating adenocarcinoma. Note the irregular arrangement of the glands and the extensive associated non-neoplastic desmoplastic stroma. **(C)** High-magnification image of a differentiated adenocarcinoma. Note the desmoplastic stroma and the marked pleomorphism in the cancer (*) relative to the trapped non-neoplastic duct (d). *Adapted from Maitra & Hruban, 2008.*

2.2.4. Precursor lesions of PDAC

The high aggressiveness and invasiveness that characterize PDAC has led to intensive research in an attempt to identify morphologically the precursor lesions whose biology and genetics should be understood in order to improve early diagnosis. The best studied precursor lesions are Pancreatic Intraepithelial Neoplasia (PanIN), Intraductal Papillary Mucinous Neoplasms (IPMN), and Mucinous Cystic Neoplasm (MCN) (Distler *et al*., 2014); being PanINs by far the most common of all precursor lesions. However, atypical flat lesions (AFL) have been lately postulated as an additional PDAC precursor (Aichler *et al*., 2012).

2.2.4.1. Pancreatic intraepithelial neoplasia - PanIN

Lesions known now as PanINs have been recognized for over a century and have been termed with a variety of names including duct hyperplasia, hypertrophy, metaplasia, and dysplasia. Recently, their significance as PDAC precursors has led to establish a universal nomenclature and histopathological classification (R. Hruban *et al*., 2001). PanINs are microscopic non-invasive epithelial neoplasms (<0.5mm) that had historically been considered to arise in the smaller pancreatic ducts, yet recent evidences points to acinar cells as the putative cell of origin based mainly on the use of GEMMs. They are characterized by mucin-containing cuboidal to columnar cells, and can be classified morphologically into three grades. PanIN-1 lesions are composed of columnar epithelial cells lacking dysplasia, with basally oriented uniform and round nuclei. PanIN-1 lesions can be flat (PanIN-1A) or papillary (PanIN-1B). PanIN-2 lesions are architecturally

more complex and they have more nuclear changes including loss of nuclear polarity, nuclear crowding, variation in nuclear size (pleomorphism), nuclear hyperchromasia, and nuclear pseudostratification. PanIN-3 lesions show high-grade dysplasia. These lesions are architecturally complex, forming papillae and cribriform structures, and in some instances clusters of cells bud off of the epithelium into the lumen of the duct. Cytologically, the nuclei in PanIN-3 lesions are enlarged, pleomorphic, and poorly oriented. Nucleoli are often prominent and mitotic figures (and even abnormal mitoses) can be seen (Hong *et al*., 2011; Hruban *et al*., 2008) (Fig. I4A).

Although PanINs are considered PDAC precursors, PanIN1 are generally regarded as benign lesions because of their ubiquitous presence in the aged population. In a recent study it has found that PanIN1 were present in 77% of the analysed cases, while PanIN2 and PanIN3 were found in lower frequencies, 28% and 4% respectively (Matsuda *et al*., 2017). Indeed, expression analysis of PanINs of different grades has shown that the expression profile of PanIN1 differs from normal ductal cells by few genes, while PanIN2, PanIN3 and PDAC gene expression is largely deregulated, suggesting that PanIN2 is the actual precursor of PDAC (Buchholz *et al*., 2005)

As expected for a precursor lesion, some proteins expressed in pancreatic cancer are also expressed in PanINs. This is the case for some mucins, particularly MUC1, MUC4, MUC5AC, and MUC6, which are expressed by PanINs (Nagata *et al*., 2007). These mucins can be used to distinguish PanINs from IPMNs, because PanINs do not display intestinal differentiation and, therefore, do not express MUC2. In addition, global analyses of gene expression have shown that a number of markers of gastric epithelial differentiation, such as pepsinogen C, MUC6, KLF4 and TFF1, are expressed in PanINs (Buchholz *et al*., 2005; Prasad *et al*., 2005).

2.2.4.2. Intraductal papillary mucinous neoplasia - IPMN

IPMNs are mucin-producing epithelial neoplasms that, by definition, involve the main and/or branch pancreatic ducts. These neoplasms are larger than PanINs (≥1 cm) and therefore can be detected by imaging. The neoplastic epithelium is usually composed of tall columnar cells with abundant apical mucin droplets. IPMNs present a papillary morphology and the degree of architectural and nuclear atypia can be dramatically heterogeneous, sometimes presenting areas of focal invasive cancer. Most common subtype of IPMN shows intestinal differentiation and is characterized by expression of MUC2 and CDX2, but not MUC1; by contrast, IPMNs with pancreato-biliary differentiation are less common, usually express MUC1 (but not MUC2 or CDX2), and are frequently associated with the presence of a carcinoma in situ (Grützmann *et al*., 2011; Maitra *et al*., 2005).

2.2.4.3. Mucinous cystic neoplasms - MCN

MCNs are distinctive mucin-producing epithelial neoplasms that are characterized by presenting an ovarian-type stroma and, in 90% of the cases, arise in women. The epithelial component of MCNs consists of columnar mucin-producing cells with varying degrees of architectural and nuclear atypia. An invasive component is present in one-third of MCNs. In contrast to IPMNs, MCNs do not communicate with the larger pancreatic ducts and differ from both IPMNs and PanINs by the presence of the ovarian-type stroma (Fernández-del Castillo, 2008).

2.2.4.4. Atypical flat lesion - AFL

In addition to the mucin-producing lesions described above, AFL have been lately described as PDAC precursors by the group of I. Esposito, as well. These consist of tubular structures lined by cuboidal cells with cytological atypia, namely enlarged nuclei with prominent nucleoli, a high nuclear–cytoplasmic ratio, and the presence of mitoses. AFLs are observed in areas of ADM and are characterized by the presence of a peculiar stroma surrounding the lesions, which is loose but highly cellular, with whorls of spindle cells that surround the tubular structures (Aichler *et al*., 2012). In the only study describing these lesions, AFLs were found to be the ones predominantly proliferative active, with 22% of them showing a Ki-67 rate between 10% and 80%, while all other lesions, including mPanIN, showed predominantly low (<10%) proliferation rates. They were found both in patients with sporadic PDAC and Familiar pancreatic cancer, as well as in the pancreas of the mutant Kras GEMMs.

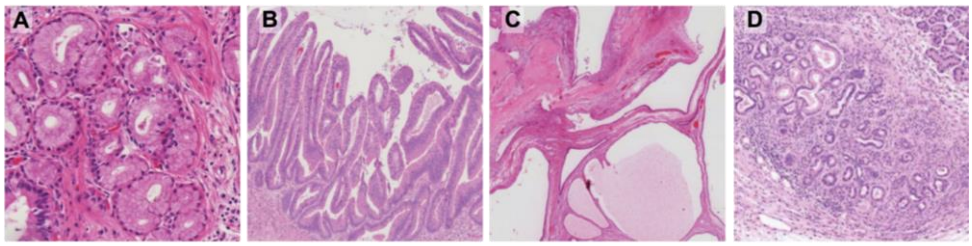

Figure I7. Precursor lesions of PDAC. Representative images of: **(A)** Pancreatic intraepithelial neoplasia (PanIN). **(B)** Intrapapillary mucinous neoplasia (IPMN). **(C)** Mucinous Cystic Neoplasia (MCN). **(D)** Atypical flat lesion (AFL). *A, B and C images were extracted from Distler et al., 2014; and D from Aichler et al., 2012.*

2.2.5. Mutational genomic landscape of PDAC

Progression from precursor lesions to invasive PDAC occurs through acquisition of mutations, epigenetic changes, and non-genetic events, including changes in the microenvironment. Although IPMN and MCN harbour their own specific

mutational features, we will focus on the mutational landscape of PanINs and how it is thought they evolve to PDAC, as it is the most relevant road to cancer in this work.

Mutational progression from PanIN to PDAC

The notion that histological progression from early neoplastic lesions to invasive cancer is related to genetic progression was first established for colon cancer (Vogelstein *et al*., 1988). By aligning specific driver mutations to cancer precursors stages, this group posited the first genetic model of tumourigenesis. Years later, a genetic model for pancreatic cancer progression was established following the same rationale (Hruban *et al*., 2000). In this model, acquisition of mutations drives the progression from preneoplastic lesions (PanINs) to invasive PDAC.

The first genetic event taking place in pancreatic carcinogenesis is an activating point mutation in the *KRAS* oncogene. Initially, it was shown that activating point mutations of *KRAS* occur in 90-95% of PDAC as well as in approximately 36%, 44%, and 87% of PanIN-1A, 1B, and 2-3 lesions, respectively (Hruban *et al*., 2008; Tada *et al*., 1996). Lately, using a more sensitive *KRAS* mutation detection technique it was shown that indeed all PanINs share a high frequency of KRAS mutations (>90%) (Kanda *et al*., 2012). In addition, telomere shortening was found in approximately 90% of PanIN-1 as well as in PanIN-2 and -3 and PDAC, which may contribute to global chromosomal abnormalities (Matsuda *et al*., 2015; Van Heek *et al*., 2002).

Following the progression model, PanIN-1 evolves to PanIN-2 through mutations of p16/*CDKN2A* (Moskaluk, Hruban, & Kern, 1997), while inactivation of the tumour suppressors *TP53*, *SMAD4*, and *BRCA2* are generally associated with progression to high-grade PanIN lesions (PanIN-3) and PDAC (Goggins *et al*., 2000; Hruban *et al*., 2008; Lüttges *et al*., 2001; Wilentz *et al*., 2002). The incidence, characterization and implications of these mutations are discussed in the following section.

Genetic alterations in PDAC

As commented, *KRAS* mutations are thought to be the earliest genetic event to occur during PDAC development, being found in 90%–95% of cancer cases as well as in PanINs (Kanda *et al*., 2012; Maitra & Hruban, 2008). Recent studies directed by the International Cancer Genome Consortium (ICGC), in which a large number of PDAC were subjected to integrated genomic analysis, confirmed a high prevalence of activating *KRAS* mutations, which were found in more than 90% of the analysed cases (P. Bailey *et al*., 2016; Biankin *et al*., 2012; Waddell *et al*., 2015; Witkiewicz *et al*., 2015). The relevance of KRAS in pancreatic cancer initiation has been underscored by the development of genetically engineered

mouse models (GEMMs) in which an activating Kras mutation is expressed from the endogenous locus under the control of a Cre recombinase driven from a pancreas-specific promoter, such as Pdx1 or Ptf1a. These mice develop the full spectrum of the human disease (Hingorani *et al*., 2003). The murine models to study pancreatic cancer will be discussed in detail in a following chapter.

KRAS belongs to a group of small guanine triphosphate (GTP) binding proteins, which oscillate between an active (GTP-bound) and an inactive (GDP-bound) state. KRAS is activated by a number of stimuli such as growth factor binding to tyrosine kinase receptors, among others. Upon ceasing of the activating stimulus, guanine-triphosphatase-activating proteins promote GTP hydrolysis to GDP and attenuate KRAS signalling (Malumbres & Barbacid, 2003). More than 90% of PDAC harbor KRAS activating mutations, located predominantly in codon 12, which inhibit the protein's ability to hydrolyze GTP, resulting in a constitutively activated molecule that signals independently of growth factor stimulation (Maitra & Hruban, 2008). Activated KRAS signals through multiple effector pathways that promote proliferation, cell survival, cytoskeletal remodeling, motility and differentiation. The best established KRAS downstream targets are the RAF-mitogen activated protein kinase (MAPK), the phosphoinositide-3-kinase (PI3K) and RAL-GDS pathways (Malumbres & Barbacid, 2003) (Fig. I8).

The important role of **RAF-MAPK** pathway in cancer has been established with the identification of activating mutations in *B-RAF* in many malignancies (H. Davies *et al*., 2002). Although they are not very common in PDAC, it was found that *B-RAF* mutations are present in one third of pancreatic cancers harbouring unaltered *KRAS*, thereby resulting in the activation of RAF-MAPK signalling in cases with wild-type *KRAS* (Calhoun *et al*., 2003). In addition, abrogation of RAS-MAPK signalling through small molecule inhibitors or with antisense technology results in growth inhibition of pancreatic cancer xenografts (Xing *et al*., 2003). Indeed, RAF-MAPK pathway has been targeted with small molecules to treat pancreatic cancer with positive results *in vitro* (Xing *et al*., 2003), yet clinical studies have shown no efficacy in patients (Infante *et al*., 2014).

The **PI3K-AKT** pathway is an essential cell survival pathway with diverse roles in tumourigenesis across multiple malignancies. The PI3K pathway is constitutively activated in most pancreatic cancers and targeting this pathway with small molecule inhibitors or genetic strategies results in growth inhibition *in vitro* and *in vivo* (Maitra & Hruban, 2008). Although RAS certainly contributes to PI3K-AKT signalling in pancreatic cancer, independent genomic events can also activate this pathway, including amplification of the *AKT2* gene in 10-20% of PDAC cases (Cheng *et al*., 1996). In addition, activating mutations and amplifications in *PIK3CA* have also been found by exome sequencing experiments (Waddell *et al*., 2015; Witkiewicz *et al*., 2015). Interestingly, combined therapies using inhibitors of the PI3K-AKT and RAF-MAPK have been

proved to be effective in vitro, leading to cell cycle arrest and apoptosis of cancer cells (Roy *et al*., 2010).

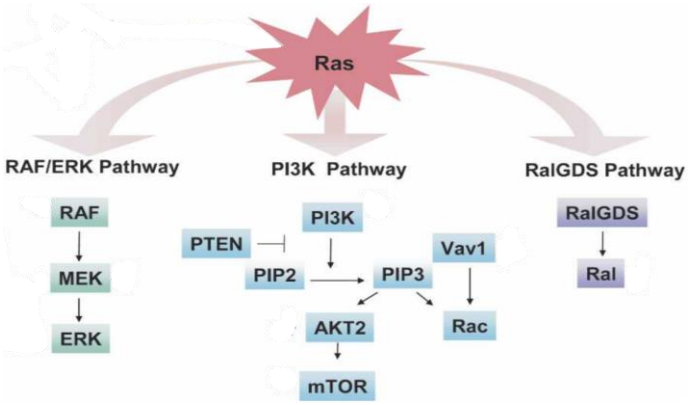

Figure I8. RAS signalling network. Ras uses a multitude of downstream effectors. Depicted are the three major signalling cascades that have been implicated in PDAC progression and mainte-nance: the Raf/Map Kinase (ERK) pathway, the PI3K pathway, and the Ral GDS pathway. *Adapted from Ying et al., 2016.*

The gene coding for **p16 (*INK4A/CDKN2A*)** is the most commonly inactivated tumour suppressor gene in pancreatic cancers. Loss of p16 function has been described to occur in more than 90% of pancreatic cancers through several mechanisms, including homozygous deletions (40%), intragenic mutation with loss of the second allele (40%), and epigenetic silencing by promoter methylation (10%–15%) (Maitra & Hruban, 2008). Recent data coming from the ICGC has confirmed the presence of CDKN2A inactivating mutations in 35% of PDAC cases, including structural variants and point mutations (P. Bailey *et al*., 2016; Biankin *et al*., 2012; Waddell *et al*., 2015). p16/*INK4A* belongs to the cyclin-dependent kinase (CDK) inhibitor family and inhibits cell cycle progression by inhibiting CDK4/6-mediated phosphorylation of RB (Kim & Sharpless, 2006).

Inactivation of the ***TP53*** gene has been described in approximately 50%–75% of pancreatic cancers through intragenic mutation combined with loss of the second allele. *TP53* loss occurs almost exclusively in the most advanced lesions (PanIN-3 or carcinoma in situ) (Hruban, 2000; Maitra & Hruban, 2008). *TP53* inactivating events including mutations and structural variants has been recently confirmed by the ICGC in 74% of PDAC cases (P. Bailey *et al*., 2016; Biankin *et al*., 2012; Waddell *et al*., 2015). P53 is a major regulator of responses to stress: under normal conditions it is functionally inactive due to its rapid degradation by the ubiquitin ligase MDM2; upon stress (including DNA damage or oncogenic signalling) MDM2-driven degradation is impaired and p53 accumulates, gaining full competence in transcriptional activation. p53 targets include cell cycle inhibitors and pro-apoptotic proteins, which results in cell-cycle arrest, senescence or apoptosis. Loss of p53 function allows cells to survive and divide despite the presence of damaged DNA, leading to the accumulation of additional

genetic abnormalities (Vousden & Lane, 2007). In murine models of PDAC, the point mutation R172H in *Trp53* gene confers gain-of-function properties to p53 that result in chromosomal instability and widely metastatic cancer (Hingorani *et al*., 2005). Recently, it has been described that sustained expression of $p53^{R172H}$ mediates PDAC pro-metastatic potential through the platelet-derived growth factor receptor b (PDGFRb), which is both necessary and sufficient to mediate these effects (Weissmueller *et al*., 2014).

The ***SMAD4*** gene was initially found to be inactivated in approximately 55% of pancreatic cancers, either by homozygous deletion (30%) or by intragenic mutations and loss of the second allele (25%). As commented earlier, this gene as well as *TP53* are usually inactivated in high-grade PanINs, and therefore these two events are considered to occur late in the linear progression model for PDAC (Hruban *et al*., 2000; Maitra & Hruban, 2008). Again, recent data from ICGC has confirmed that 31% of PDAC harbour *SMAD4* genetic alterations (P. Bailey *et al*., 2016; Waddell *et al*., 2015). SMAD4 is a transcriptional regulator that plays a critical role in the transforming growth factor-β (TGF-β) pathway. TGF-β is a cytokine that binds to serine/threonine kinase receptors, which propagate the signal by phosphorylating SMAD transcription factors (i.e. SMAD2 and 3) that shuttle to the nucleus where they form a complex with Smad4, activating a variety of downstream targets (Massagué, 2008). The TGF-β pathway has profound growth-inhibitory effects by regulating the expression of specific target genes; therefore, loss of Smad4 in pancreatic cancer cells, which abrogates Smad-dependent TGF-β signalling, provides a selective growth advantage (P. M. Siegel & Massagué, 2003).

In addition to the genetic alterations of these "classic" genes (*KRAS*, *TP53*, *CDKN2A* and *SMAD4)*, which have been widely studied and characterized, next generation sequencing (NGS) has facilitated the study of additional genetic players. Jones *et al*. were the first to interrogate the PDAC genome globally (Jones *et al*., 2008), and they found that nearly all tumours harboured mutations in Hedgehog/Wnt/Notch signalling components. Another study analysing more than 100 PDAC determined that one fifth of PDAC genomes harbour mutations and copy number aberrations in genes involved in axon guidance a signalling pathway that provides attractive and repulsive cues in axon development (Biankin *et al*., 2012). In addition, this study identified several new genetic players that are mutated at low frequency (generally below 5%) in PDAC, including *MLL3, TGFBR2, ARID1A, EPC1, ARID2, SF3B1, ATM* and *RNF43.*

The resolution of the first genomic analyses was limited by the fact that PDAC are often highly desmoplastic. By needle microdissection to enrich for epithelial tumour cells prior to exome sequencing, Witkiewicz *et al*. actually found mutation rates of PDAC were higher compared to Biakin *et al*. Their increased detection limit allowed identifying novel recurrent mutations (still at or below

5%) in *BCLAF1, IRF6, FLG, AXIN1, GLI3, PIK3CA,* and *RBM10* (Witkiewicz *et al*., 2015). More recent genomic studies analysing more than 450 PDAC cases have characterized novel mutations, being able to define 10 pathways -some already known to be altered and some new ones- that are commonly affected in PDAC, including KRAS, TGF-β, WNT, NOTCH, ROBO/SLIT signalling, G1/S transition, SWI-SNF, chromatin modification, DNA repair and RNA processing (P. Bailey *et al*., 2016). Overall, these studies have revealed a striking mutational heterogeneity in PDAC and have confirmed that beyond the "classic four" genetic players, recurrent mutations in PDA generally occur in rare subsets.

In addition to mutations activating oncogenes or deletion of tumour suppressors, **telomere erosion** also plays an important role in PDAC initiation and progression. Indeed, it is one of the earliest demonstrable genetic aberrations in pancreatic cancer, with >90% of PanIN lesions presenting it compared to normal ducts (Matsuda *et al*., 2015; Van Heek *et al*., 2002). Telomeres consist of repetitive DNA sequences that associate with shelterins, proteins that confer stability to chromosomes during cell division. Loss of telomeres renders chromosome ends highly recombinogenic, which may result in amplifications and deletions (Shay & Wright, 2010). Cells with critically short telomeres activate a p53-dependent DNA damage response that prevents from immortalization and transformation of these cells. However, p53 is lost only in 50-75% of PDAC cases which raises the possibility that other p53 pathway components involved in the telomere-induced checkpoint responses are neutralized in a subset of pancreatic cancers (Hezel *et al*., 2006).

2.2.6. Progression models of PDAC development

2.2.6.1. The "classical" model

The "classical model" for pancreatic cancer progression states that tumours arise from histologically well-defined precursor lesions through the accumulation of multi-step genetic alterations (Hruban *et al*., 2000; Maitra & Hruban, 2008). In this regard, PanINs are considered to be the most common precursor lesion as they are frequently found in biopsies from patients presenting PDAC. Classically, the model has proposed that PanINs arise in small ducts through mutations that initiate the neoplastic process (Maitra & Hruban, 2008). However, several studies in mice have shown that acinar and centroacinar cells could rather be the cell of origin of PanINs (Aichler *et al*., 2012; Bailey *et al*., 2014; Carrière *et al*., 2007; De La O *et al*., 2008; Esposito *et al*., 2007; Guerra *et al*., 2007, 2011; Habbe *et al*., 2008; Kopp *et al*., 2012; Morris IV *et al*., 2010). Regardless of the compartment where they originate, PanINs are proposed to progress in a linear fashion through the sequential stepwise accumulation of mutations as already discussed: *KRAS* mutations would initiate transformation and loss of p16/*INK4A*/*CDKN2A*, *TP5*3 and *SMAD4* would be the cause of progression first to

more dysplastic lesions (PanIN2 and PanIN3) and eventually to invasive cancer. The major evidence that led to the development of the linear progression model for PDAC is the observation that PanINs harbor many of the genetic alterations that are found in invasive pancreatic cancer (Fig. I9A).

2.2.6.2. The "alternative" model

The linear progression model initially proposed (Hruban *et al*., 2000) has been questioned given a number of limitations that may not have been appropriately taken into account by the scientific community, leading to a rapid establishment of a "dogma" model. These limitations have been described elsewhere (Notta *et al*., unpublished; Real, 2003; Real *et al*., 2008)..The existing evidence supporting PanIN-1 to PDAC progression considering the prevalence of genetic changes does not allow concluding about the sequence in which a given genetic alteration occurs, and could actually be interpreted differently. Therefore, the alternative model suggests that *KRAS* mutations might favour the appearance of dysplasia and tumour progression only when occurring in cells harbouring loss of heterozygosity (LOH) in a crucial tumour suppressor gene, such as *INK4A* or *TP5*3. On the other hand, *KRAS* mutations occurring in normal cells might lead to growth arrest by activation of senescence. This would mean that only PanIN-2/3 would progress into PDAC while PanIN-1, presenting only *KRAS* mutations, would undergo cell cycle arrest (Real *et al*., 2008). The proposed model is supported by results obtained using global transcriptome analysis of human PanINs, showing that the expression profiles of PanIN-1 lesions were similar to those of normal ducts, while PanIN-2 and 3 cluster together with PDAC samples (Buchholz *et al*., 2005). Moreover, it has been firmly established using *in vitro* and *in vivo* studies the existence of oncogene-induced senescence, which would function as a protective mechanism against tumour progression in cells that are subjected to oncogenic stress (Collado *et al*., 2005). In fact, in a mouse model of PDAC expressing oncogenic Kras in the pancreas, it has been shown that PanINs were positive for senescence markers while PDAC was negative (Caldwell *et al*., 2012; Collado *et al*., 2005) (Fig. I9B).

The alternative model is in agreement with a recent study analysing mutations, DNA copy number changes, and genomic rearrangements of human PDAC genomes using whole genome sequencing (Notta *et al*., 2016). This study has revealed that: first, pancreatic carcinogenesis does not necessarily develop through a particular sequence of genetic alterations (*KRAS* followed by *CDKN2A*, then *TP53* and *SMAD4*); and second, that the evolutionary trajectory of pancreatic cancer progression is not gradual, or in other words, that each alteration is not acquired independently as initially proposed. The study shows that 45% of PDAC displays changes in copy number consistent with polyploidization. In addition, two thirds of analysed PDAC carry complex

rearrangement patterns associated with mitotic errors, pointing to genomic instability as the principal cause of malignant progression. The study also shows that a single event of chromothripsis can inactivate *en bloc* the tumour suppressors *CDKN2A*, *TP53* and *SMAD4*, being this a major transforming event contributing to malignancy. This rare event could confer a cell both invasive and metastatic properties, thus accounting for the very short latency between the birth of the invasive clone and the acquisition of its ability to metastasize (Notta *et al*., 2016).

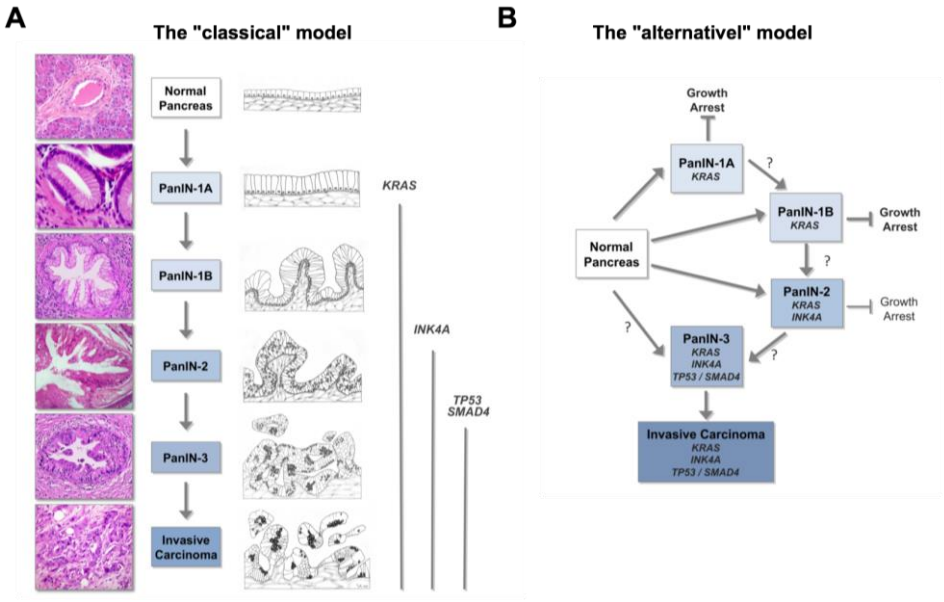

Figure I9. Model of PDAC progression. **(A)** Classical model showing the linear progression from low to high-grade PanIN to carcinoma with the mutations associated in each step. **(B)** Alternative model showing that PDAC may arise from advanced lesions and that low-grade PanIN act as tumour suppressor events that allow growth arrest of neoplastic cells. *Adapted from Pinho, 2011.*

2.2.7. Genetically engineered mouse models to study pancreatic cancer

The first approaches to generate mouse models of pancreatic cancer started in the late 1980s and they were based on the transgenic expression of oncogenes that resulted in neoplastic processes different from the ones observed in the human setting (Ornitz *et al*., 1987; Quaife *et al*., 1987). The first GEMM closely resembling the human disease was established in 2003 (Hingorani *et al*., 2003). This model is based on a strain of mice expressing a conditional *Kras*^{G12D} mutant allele silenced by a floxed transcriptional STOP cassette (Lox-Stop-Lox or LSL) inserted upstream of the targeted Exon1 (LSL-*Kras*^{G12D} mice). Removal of the LSL cassette by expression of Cre recombinase allows expression of oncogenic

Kras^{G12D}. When these mice are crossed with those expressing Cre recombinase under the control of the *Pdx1* or *Ptf1a* regulatory sequences, mice develop PDAC (Hingorani *et al*., 2003). Such compound mutant mice (*Pdx1*$^{+/Cre}$;LSL-*Kras*^{G12D} and *Ptf1a*$^{+/Cre}$;LSL-*Kras*^{G12D}) develop PanIN with complete penetrance and a subset of older mice develop PDAC (Fig. I10). The PanINs that develop in these mice resemble those of patients in that they express mucins, cytokeratin-19, Cyclooxygenase (Cox)-2, MMP-7, and Hes1 (Hingorani *et al*., 2003). This GEMM supported the PanIN-to-PDAC model of pancreatic cancer progression described by Hruban *et al*., 2000.

As discussed earlier, human PanINs are believed to progress to PDAC following inactivation or point mutation of p16/*CDKN2A*, *TP53*, and *SMAD4* and other regulators of TGFb pathway (Maitra & Hruban, 2008). Studies of GEMMs incorporating these additional mutant alleles to the expression of mutant *Kras* in the pancreatic compartment have supported the proposed model of progression to PDAC. Specifically, mice harbouring monoallelic and biallelic loss of the *p16*^{Ink4a}/*p19*^{Arf} and *Trp53* genes (Aguirre *et al*., 2003; Bardeesy *et al*., 2006), the concomitant expression of dominant-negative forms of p53 (Hingorani *et al*., 2005), or the ablation of the type II TGF-β receptor (Ijichi *et al*., 2006) develop invasive and metastatic PDAC (Fig. I10).

In the above described mice, mutant *Kras* expression is activated in progenitor cells during pancreas organogenesis and, therefore, all cells in the adult pancreas express the mutant allele, not allowing to determine the cell of origin of preneoplastic lesions and PDAC. In this regard, attempts to induce PanINs from ductal cells by regulating expression of *Kras* with the promoters of the ductal markers cytokeratin-19 (Brembeck *et al*., 2003), Hnf1b (Diersch *et al*., 2015), or Sox9 (Kopp *et al*., 2012) did not lead to PanINs or PDA. In contrast, a GEMM harbouring an active Kras carrying a G12V mutation expressed under the Elastase promoter during late embryonic development, and therefore expressed in the acinar compartment, reproduced the development of PanIN lesions as well as invasive PDAC with an incidence and latency similar to those observed in mice expressing *Kras*^{G12D} from pancreatic progenitors (Guerra *et al*., 2007). These observations, together with other studies, provided evidence that PanINs and PDAC can originate from acinar cells (De La O *et al*., 2008; Guerra *et al*., 2007; Habbe *et al*., 2008) and that ductal cells appear to be more resistant to mutant KRas-induced transformation. Indeed, these results support the model described above where acinar-to-ductal metaplasia might be the earliest pancreatic lesion and, as such, the precursor of low-grade PanINs (Storz, 2017).

As commented, pancreatitis is a risk factor for PDAC development and this observation has been harnessed in mouse models to accelerate pancreatic carcinogenesis. Actually, when Kras is activated in acinar cells in the post-natal pancreas of 8-week-old mice, cells are refractory to the development of PanINs

and PDAC (Guerra *et al*., 2007). Only when mice are subjected to chronic pancreatitis neoplastic events, both PanIN and PDAC, are observed (Guerra *et al*., 2007, 2011). In addition, carcinogenesis is enhanced in mice with embryonic expression of mutant *Kras* when subjected to even a single episode of acute pancreatitis (Carrière, *et al*., 2009; Carrière, *et al*., 2011; Flandez *et al*., 2014).

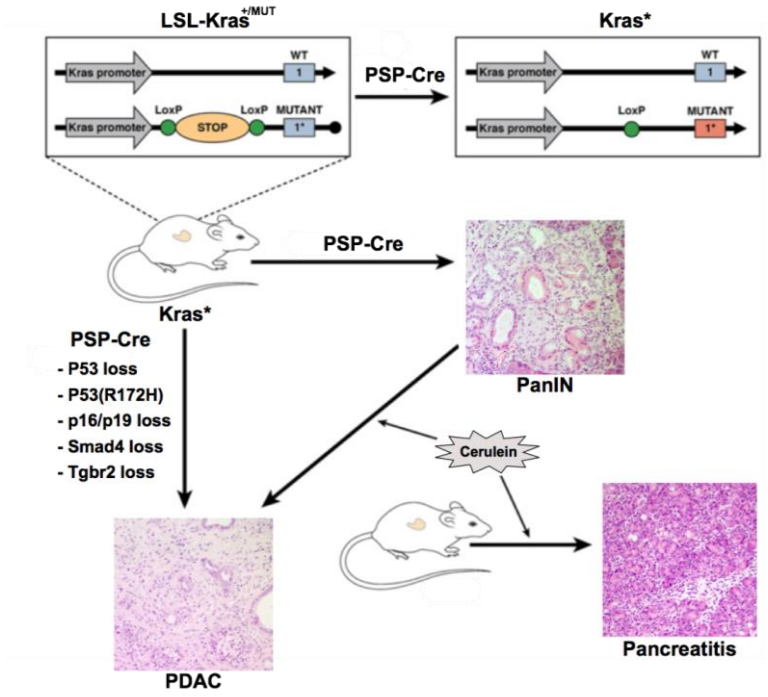

Figure I10. GEMMs for PDAC study. Expression of Cre recombinase, regulated by pancreas-specific promoters (PSP), leads to conditional activation of mutant Kras allele in the pancreas; which induce development of PanINs. PanINs can progress to PDAC in cooperation with loss of tumour suppressor genes, including p53, p16Ink4a/p19Arf, Smad4, and Tgfbr2. Nongenetic events, such as induction of pancreatitis by cerulein, cooperate with oncogenic Kras in PanINs to promote progression to PDAC. *Adapted from Pérez-Mancera, et al., 2012*

In addition to the known genes whose mutation contributes to PDAC progression, it is very likely that PDAC can progress through alterations in other genes whose role in PDAC progression has not been described yet. The use of transposons has allowed uncovering those genes through an agnostic approach. Transposons are DNA transposable elements that are recognized by transposases, which can mobilize the transposons along the genome in an "excise and jump" mechanism. There are 2 well-described transposition systems that can promote the development of solid tumours: Sleeping Beauty and PiggyBac. They have gene-trapping features that capture upstream exons to inactivate potential tumour suppressor genes (loss-of-function activity). The transposons

can also include a strong promoter element to ectopically activate the expression of potential proto-oncogenes or dominant-negative forms of tumour suppressor genes (gain-of-function activity). The tropism of insertional mutagenesis is controlled by tissue-specific transposase alleles. Although this biallelic system can promote neoplasms in certain tissues by itself (such as in colon), in the pancreas it was observed that the *Kras*^{G12D} allele was required to sensitize the mice for the development of PDAC. A study using Sleeping Beauty system showed that this technology promoted early lethality from PDAC, and analyses of common insertion sites in this screen revealed that the TGF-β and Rb/p16^{Ink4a} pathways were affected, confirming the relevance of this approach (Pérez-Mancera *et al*., 2012). In addition, this study identified the deubiquitinase USP9X as a major tumour suppressor in pancreatic carcinogenesis.

3. GATA TRANSCRIPTION FACTORS

3.1. The origin and molecular structure of the GATA family

GATA proteins are a family of transcription factors that are involved in a variety of physiological and pathological processes. All GATA members contain a highly conserved DNA binding domain consisting of two zinc fingers of the motif Cys-X2-Cys-X17- Cys-X2-Cys (Tsai *et al*., 1989) that directs binding to the nucleotide sequence element (A/T)GATA(A/G) (Ko & Engel, 1993). Although all GATA proteins contain two Zn finger domains, a number of reports have demonstrated that only the C-terminal zinc finger and an adjacent basic domain are necessary for specific DNA binding in vitro (Omichinski *et al*., 1993; Visvader *et al*., 1995).

GATA-like transcription factors have been studied in invertebrates such as D. melanogater and six different GATA members have been identified in vertebrates displaying more than 70% conserved in the Zn finger domain, while the sequences of the amino-terminal and carboxyl-terminal domains exhibit lower similarity (Morrisey *et al*., 1997). According to traditional phylogenetic analysis and tissue expression profiles, GATA proteins can be divided into two subfamilies: GATA1/2/3 are expressed in hematopoietic cell lineages and are essential for differentiation of erythrocytes and megakaryocytes, proliferation of hematopoietic stem cells and development of T lymphocytes (Shimizu & Yamamoto, 2016). However, their expression is not limited to hematopoietic lineages, as they also play an important role in the development of brain, spinal cord and inner ear (George *et al*., 1994; Lilleväli, *et al*., 2004; Nardelli, *et al*., 1999). In contrast, GATA4/5/6 are considered the mesodermal-endodermal group as these proteins were initially found to be expressed in tissues with this embryonic origin, such as heart, gut, liver and gonads (Lavemiere *et al*., 1994; Molkentin, 2000). However, such histotypic classification is no longer supported by the fact that both "hematopoietic" and "endodermal" GATAs are involved in the development of tissues with a different embryonic origin than originally described (i.e. GATA4 and GAT6 in central nervous system or GATA1 in testis, Fig. I11). Yet, a study analysing the phylogenetic relationship between GATA proteins revealed that the two subfamilies cluster separately, indicating that this classification is consistent with their initially described functions (He *et al*., 2007).

3.2. Gata4 and Gata6 as main factors controlling pancreatic organogenesis

The first evidence of GATA4 and GATA6 being expressed in the pancreas appeared in a study where mRNA expression was assessed in all human tissues

by northern blotting (E. Suzuki *et al*., 1996). It was found that in adult, GATA6 was expressed at high levels in heart, ovary, lung and pancreas. Conversely, GATA4 was expressed at highest levels in testes, pancreas, ovary and heart, showing that both GATA members are expressed in pancreas, ovary and heart.

Although GATA4 and GATA6 transcripts could not be detected in human embryonic pancreas (E. Suzuki *et al*., 1996), many reports subsequently showed that these transcription factors play a key role in mouse pancreas development.

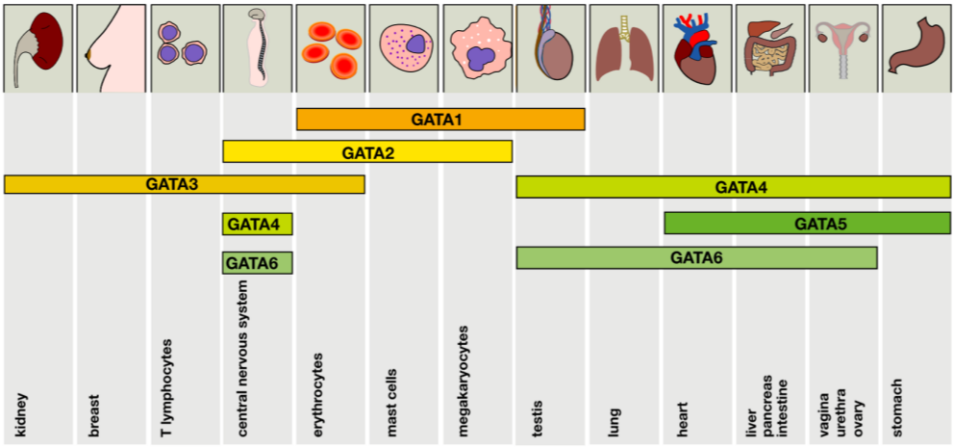

Figure I11. GATA factors in tissue development. Schematic representation showing which GATA factors are required for the development of a variety of tissues. As depicted, the hystotypic classification dividing them as "hematopoietic" (GATA1/2/3) or "endodermal-mesodermal" (GATA4/5/6) is no longer supported as GATA4/6 play a role in the development of the central nervous system (ectoderm), and GATA1 is needed for the development of testis (endoderm). *Extracted from Lentjes et al., 2016.*

The specific functions of Gata4 and Gata6 in murine pancreas development have not been precisely determined since embryos lacking Gata4 or Gata6 are lethal before pancreas formation. Gata4-null mouse embryos are arrested in development between E7.0 and E9.5 due to severe developmental abnormalities, including lack of a primitive heart tube, lack of foregut and a development partially outside the yolk sac (Kuo *et al*., 1997; Molkentin *et al*., 1997). Gata6-null embryos are lethal shortly after implantation (E5.5) due to a defect in extraembryonic tissues that, in turn, affect epiblast development (Koutsourakis *et al*., 1999). Experiments of tetraploid embryo complementation to avoid early lethality showed a complete absence of the ventral but not the dorsal pancreas in *Gata4*$^{-/-}$ embryos. Likewise, *Gata6*$^{-/-}$ embryos displayed a similar, although less dramatic phenotype, suggesting a critical role for multiple Gata factors at the earliest stages of ventral pancreas development (Watt *et al*. 2007).

Pancreas-specific deletion of *Gata4* and *Gata6* mediated by *Pdx1*-driven expression of Cre recombinase showed that both genes control pancreas organogenesis. Deletion of either *Gata4* or *Gata6* results in mild pancreatic defects that resolve postnatally. However, simultaneous deletion causes severe pancreatic agenesis that is attributed to disruption of pancreatic cell proliferation, defects in branching morphogenesis and a subsequent failure to induce differentiation of progenitor cells. Interestingly, deletion of a single Gata6 allele in a Gata4-null background severely reduces pancreatic mass, while one functional Gata4 allele in a Gata6-null background is sufficient for normal pancreatic development, indicating differential contributions of Gata factors to pancreas formation. These studies placed Gata factors at the top of the transcriptional network hierarchy controlling pancreas organogenesis (Carrasco *et al*., 2012; Xuan *et al*., 2012).

Despite these studies unequivocally showing that Gata factors are crucial for pancreas development (Rodríguez-Seguí *et al*., 2012), their specific roles in the exocrine and endocrine components have been a matter of discussion and controversy. Studies performed in human tissue describing that both GATAs are expressed in the pancreas did not address whether expression was in endocrine or exocrine compartment (E. Suzuki *et al*., 1996). In mice, Gata4 was found to be expressed in the exocrine pancreas (Decker, *et al*., 2006; Ketola *et al*., 2004; Nemer, *et al*., 2003; Ritz-Laser *et al*., 2005). In addition, some reports using RIN1046-38 and glucagonoma and insulinoma cell lines also attributed Gata4 a role in the endocrine lineage (Swift *et al*., 1994), with the ability of activating Glucagon expression (Ritz-Laser *et al*., 2005). Similar results were described for Gata6, with some reports describing that it is expressed in the exocrine pancreas (Nemer *et al*., 2003; Ritz-Laser *et al*., 2005), and others proposing that Gata6 is restricted to the endocrine compartment (Decker *et al*., 2006; Ketola *et al*., 2004). However, the conclusions drawn from the later studies may not be adequated as they were based on:

1) *In situ* hybridisation experiments showing that Gata6 mRNA was expressed at E12.5 in the pancreatic epithelium in the same regions as the endocrine transcription factor Nkx2.2 and the marker of endocrine progenitors Ngn3 (Decker *et al*., 2006). However, detection of Gata6, Nkx2.2 and Ngn3 transcripts in similar regions at E12.5 does not imply that Gata6 cannot be expressed in the adult exocrine pancreas.

2) IHC stainings showing that Gata6 was exclusively expressed in the Islets of Langerhans in the adult murine pancreas (Ketola *et al*., 2004). However, the Gata6 antibody they used was considered to be not reliable in subsequent studies, including the one from Decker *et al*. 2006.

A recent study performed by Martinelli *et al*. in our laboratory clarified the role of Gata6 in adult pancreas. In this study, it was shown by IHC that Gata6 is expressed in the adult pancreas in acinar, ductal and endocrine cells. Although antibody reliability had been a controversial issue, the fact that Gata6 signal was lost in Gata6-null pancreas indicated high specificity of the antibody.

In this study, the role of Gata6 in the adult pancreas was investigated by deleting the gene specifically in the pancreas during embryo development through Cre Recombinase expressed under the promoter of *Ptf1a,* which is active starting around E9.5. Histology of Gata6-null pancreata of 8-week-old mice looked normal. However, transcripts coding for digestive enzymes and regulators of the acinar programme -such as Ptf1a, Rbpjl or Mist1- were largely downregulated, indicating that Gata6 is required for complete acinar differentiation. Mislocalization of the apical marker Muc1 in *Gata6*$^{-/-}$ pancreata indicated that Gata6 is also necessary for establishment of acinar cell polarity. In addition, Gata6-null pancreata of 30-week-old mice presented with massive loss of acinar cells and replacement by fat, increased acinar apoptosis and proliferation, ADM and adipocyte transdifferentiation, indicating that Gata6 is necessary to maintain acinar cell identity in the adult pancreas.

The deleterious effect of Gata6 deletion in acinar cells and the fact that neither mouse weight nor response to glucose administration were different in Gata6-null mice indicated that Gata6 plays a crucial role in the exocrine, but not the endocrine, pancreas.

The role of GATA4 and GATA6 in human pancreas development is evidenced by the fact that inactivating mutations in these genes result in pancreas agenesis. Regarding GATA6, there are several reports showing that damaging mutations cause pancreatic defficiencies. The study with the largest number of cases analysed determined that 15/27 cases presenting pancreatic agenesis where due to heterozygous mutations in GATA6 (Allen *et al*., 2012). In addition, other studies (most of them case-report studies) have shown that *GATA6* mutations can lead to a broad spectrum of phenotypes, ranging from adult-onset diabetes without exocrine insufficiency, to pancreatic malformations/hypoplasia (causing neonatal diabetes), as well as pancreatic agenesis(Bonnefond *et al*., 2012; Chao *et al*., 2015; De Franco *et al*., 2013; Eifes *et al*., 2013; M. Gong *et al*., 2013; Stanescu *et al*., 2015; Suzuki *et al*., 2014; Yau *et al*., 2017; Yorifuji *et al*., 2012). In the case of GATA4, it has been found that inactivating mutations and deletions cause neonatal or childhood-onset diabetes, with different degrees of exocrine insufficiency (Shaw-Smith *et al*., 2014). Although one report has also attributed a mutation in *GATA4* causing heart defects together with pancreas agenesis, the presence of the same mutations in other individuals was only associated to cardiac defects with no signs of pancreas affectation, indicating that the two

pathologies were attributable to two independent events, and that mutation of *GATA4* might not be the cause for the pancreas affectation (D'Amato *et al*., 2010).

In addition to the already referred study, mutations in *GATA4* may cause cardiac developmental defects (Garg *et al*., 2003; Pehlivan *et al*., 1999). Actually, many studies have attributed a crucial role of GATA4 in heart development both in human and mouse (Borok & Papaioannou, 2015; Holtzinger & Evans, 2005; Kuo *et al*., 1997; Molkentin *et al*., 1997; Pu *et al*., 2004; Watt *et al*., 2004; Zhao *et al*., 2008). In the case of GATA6, although it is not necessary for early heart development (Zhao *et al*., 2005), it also plays a role in cardiogenesis as some mutations -also causing pancreatic defects- result in alterations in heart development (Eifes *et al*., 2013; M. Gong *et al*., 2013). Altogether, these studies highlight the importance of these transcription factors in the development of multiple organs.

3.3. GATA6 in PDAC

The role of GATA6 in pancreatic carcinogenesis has also been a field of discussion and controversy. Based on the occurrence of GATA6 gains/amplifications in some tumours, an oncogenic role was proposed for GATA6 in PDAC (Fu, *et al*., 2008; Kwei *et al*., 2008). This notion was supported by *in vitro* assays showing that siRNA-mediated knockdown of GATA6 in pancreatic cancer cell lines with amplification led to reduced cell proliferation, cell cycle progression, and colony formation (Kwei *et al*., 2008); and that forced overexpression of GATA6 in MiaPaca2 cells resulted in increased proliferation and growth in soft-agar (Fu *et al*.. 2008). However, the number of tumours studied was relatively small in both studies, and *in vitro* assays did not provide mechanistic insights on the putative role of GATA6 as an oncogene. In addition, another study showed that high GATA6 levels were found in well-differentiated tumours and were associated with better patient outcome (Zhong *et al*., 2011), suggesting that GATA6 function in pancreatic cancer progression could be more complex than originally proposed.

Recently, and contrary to what had been published earlier, studies using mouse models of pancreatic cancer have attributed Gata6 a tumour suppressor role. It has been shown that Gata6 deletion renders acinar cells more sensitive to KrasG12V, thereby accelerating tumour progression. In addition, Gata6 expression is lost in KrasG12V-driven PDAC, specifically in poorly differentiated tumours. Mechanistically, it was proposed that Gata6 exerts its tumour-suppressive effect through the promotion of cell differentiation, the suppression of inflammatory pathways, and the direct repression of the cancer-related EGFR pathway, which is upregulated in the normal and preneoplastic Gata6-null pancreas as well as in GATA6-silenced human PDAC cells (Martinelli *et al*., 2015). Other reports have also linked loss of Gata6 activity to dedifferentiation of acinar

cells and enhanced tumourigenesis in the KrasG12D mouse model, in this case through nicotine administration (Hermann *et al*., 2014). The mechanisms through which GATA6 promotes differentiation of tumour cells were further dissected by Martinelli *et al*., 2016. They found that GATA6 inhibits the epithelial–mesenchymal transition (EMT) *in vitro* and cell dissemination *in vivo* through a unique pro-epithelial and anti-mesenchymal function. Furthermore, they observed that GATA6 is commonly lost in tumours with altered differentiation, which have acquired a basal-like molecular phenotype. In humans, presenting with a basal-like GATA6-low tumour is linked to shorter survival and a distinctly poor response to adjuvant 5-fluorouracil (5-FU)/leucovorin (Martinelli *et al*., 2016).

Experiments where PDAC genomes have been analysed by next generation sequencing (NGS) technology have confirmed that *GATA6* is amplified in a subset of tumours, and in some studies this amplification has been linked to tumours with better outcome (P. Bailey *et al*., 2016; Murphy *et al*., 2016; Waddell *et al*., 2015). Interestingly, GATA6 has also been found to be hypermethylated in another subset of tumours with basal-like features presenting the poorest survival (P. Bailey *et al*., 2016), supporting its tumour suppressor role in human PDAC.

HYPOTHESIS & AIMS

During embryo development, Gata4 and Gata6 play a crucial role in pancreas organogenesis. In the adult pancreas Gata6 is required for acinar cell hoemostasis and acts as a tumour suppressor in pancreatic carcinogenesis, yet the role of Gata4 has not been studied.

We hypothesize that Gata4 might also be an important regulator of pancreas homeostasis and we aim to investigate whether it also plays a role in pancreatic tumourigenesis. Therefore, in this work we set the following objectives:

1. To define the role of Gata4 in pancreas homeostasis.

2. To define the role of Gata4 during pancreatic damage and recovery.

3. To dissect the function of Gata4 during pancreatic carcinogenesis in a mouse model of pancreatic cancer.

4. To analyse whether Gata4 plays a role in human pancreatic carcinogenesis and whether it functions as a tumour suppressor or an oncogene.

MATERIALS & METHODS

1. MOUSE STRAINS

Mouse strains used in the experiments were in a predominant C57BL/6 background with the following genetic modifications (alone or in combination): *Ptf1a*$^{+/Cre}$ (Chris Wright, Vanderbilt University; Kawaguchi *et al*., 2002), *Gata4*$^{lox/lox}$ (Jackson Laboratory; Watt, *et al*., 2004), *Kras*$^{+/LSL\text{-}G12Vgeo}$ (Mariano Barbacid, CNIO; Guerra *et al*., 2003), *Ptf1a*$^{+/CreERT2}$ (Chris Wright, Vanderbilt University; Pan *et al*., 2013), *Reg3b*$^{-/-}$ (Juan Iovanna, Centre de Recherche en Cancérologie de Marseille; Lieu *et al*., 2006), *Trp53*$^{lox/lox}$ (Jackson Laboratory; Donehower *et al*., 1992), *Rosa26*$^{YFP/YFP}$ (Philippe Soriano, Icahn School of Medicine at Mount Sinai; Soriano, 1999). Mice were bred to generate the different strains used in the study (Table M1) and maintained under sterile and pathogen-free conditions at the animal room of CNIO. Experiments were approved by the Animal Ethical Committee of Instituto de Salud Carlos III and performed following guidelines for Ethical Conduct in the Care and Use of Animals.

Strain	Short name	Procedures/Experiments
Wild-Type	WT	Acini culture (IHC), Pancreatic Duct Ligation
Ptf1a$^{+/Cre}$; ***Gata4***$^{\mathbf{lox/lox}}$	*Gata4*$^{P\text{-}/-}$	IHC, RT-qPCR, RNAseq, glucose tolerance test, mild acute pancreatitis, *in vitro* ADM, conditioned medium.
Ptf1a$^{+/Cre}$; *Kras*$^{+/LSL\text{-}G12Vgeo}$; ***Gata4***$^{\mathbf{lox/lox}}$	*Kras**;*Gata4*$^{P\text{-}/-}$	IHC, histopathological analysis, severe acute pancreatitis, RNAseq, acini culture (RT-qPCR), *in vitro* ADM, conditioned medium.
Ptf1a$^{+/CreERT2}$; *Kras*$^{+/LSL\text{-}G12Vgeo}$; *Rosa26*$^{YFP/YFP}$; ***Gata4***$^{\mathbf{lox/lox}}$	i-*Kras**;*Gata4*$^{P\text{-}/-}$	TMX administration, chronic pancreatitis (2 and 6 months), histopathological analysis.
Ptf1a$^{+/Cre}$; *Kras*$^{+/LSL\text{-}G12Vgeo}$; *Trp53*$^{lox/lox}$; ***Gata4***$^{\mathbf{lox/lox}}$	*Kras**;*Trp53*$^{-/-}$;*Gata4*$^{-/-}$	Histopathological analysis, survival.
Ptf1a$^{+/Cre}$; *Kras*$^{+/LSL\text{-}G12Vgeo}$; *Trp53*$^{+/lox}$; ***Gata4***$^{\mathbf{lox/lox}}$	*Kras**;*Trp53*$^{+/-}$; *Gata4*$^{-/-}$	Survival
Reg3b$^{\mathbf{-/-}}$	*Reg3b*$^{-/-}$	*In vitro* ADM

Table M1. **Strains** generated for this work and corresponding procedures/experiments. In all experiments, we included control mice harbouring wild-type alleles of those represented in bold.

2. IN VIVO PROCEDURES

2.1. Recombination in i-*Kras*;Gata4*$^{P\text{-}/-}$ mice

Recombination was achieved by administrating 3 doses of 4-Hydroxytamoxifen (TMX) via gavage to 8-10 week old mice. On days 1 and 3, mice received 10mg

TMX diluted in 100µl of a solution containing 10% EtOH, 90% Saline; and at day 5, mice received 5mg of TMX in 50 µl of the same solution. After the last dose of TMX, mice were free of treatment at least for 1 week before proceeding with further experimental manipulation.

2.2. Caerulein-induced acute pancreatitis

Induction of a mild acute pancreatitis

Acute pancreatitis was induced by seven hourly intraperitoneal injections of caerulein (50µg/kg); saline-treated mice were used as experimental controls. Mice were sacrificed by cervical dislocation at 24 hours, 48 hours, 7 days and 14 days after the treatment. The pancreas was quickly collected: one piece was flash frozen and stored at -80 ºC for subsequent RNA analysis, and the rest of the pancreas was fixed for subsequent histological analysis. A minimum of 3 mice/group were included as saline controls and 6 mice/group were used in each pancreatitis timepoint.

Induction of a severe acute pancreatitis

A severe acute pancreatitis was induced by seven hourly intraperitoneal injections of caerulein (50µg/kg) during two non-consecutive days, with 24 hours intermission. Pancreatitis was induced at "early" and "late" timepoints in cohorts of 20-week-old and 40-week-old mice, respectively. The "early" cohort included *Kras** (n=4) and *Kras*;Gata4*$^{P-/-}$ (n=8) mice; and the "late" cohort included *Kras** (n=7) and *Kras*;Gata4*$^{P-/-}$ (n=6) mice. All mice were sacrificed at 1 year of age by cervical dislocation, and the pancreas was collected for histological analysis.

2.3. Caerulein-induced chronic pancreatitis

Chronic pancreatitis was induced by daily injection of one dose of caerulein (5µg/mouse in 100µl of saline). In order to test whether chronic administration of caerulein could accelerate PDAC formation, a cohort of 10-12 week-old mice including i-*Kras** (n=6) and i-*Kras*;Gata4*$^{P-/-}$ (n=9) was treated during 6 months and allowed to further age for 3 months until reaching 1 year of age. In order to test whether chronic administration of caerulein could accelerate PanIN development, a cohort of 10-12 week-old mice including i-*Kras** (n=4) and i-*Kras*;Gata4*$^{P-/-}$ (n=6) was treated for 2 months. At the end of the protocol, mice were culled by cervical dislocation and the pancreas was collected for histological analysis.

2.4. Intrapancreatic adenoviral administration

Mice were appropriately anesthetized and a small incision was performed in the skin and peritoneum at the upper left quadrant to expose the pancreas. Adenovirus ($1x10^9$ plaque-forming units [pfu] in 50 µl of saline) encoding either GFP (Ad-EGFP) or IL-17A (Ad-IL-17A) (Schwarzenberger *et al*., 1998) were injected directly into multiple sites of the pancreas of 10 week-old mice. A cohort of *Kras** (n=5) and *Kras*;Gata4*$^{P-/-}$ (n=5) mice were injected with Ad-EGFP, and another cohort with the same number of mice was injected with Ad-IL-17A. Mouse serum was collected prior to virus infection, 2 weeks post-infection, and 6 weeks post-infection, before sacrifice. Mice were sacrificed by cervical dislocation and pancreas was quickly processed: a piece of tissue was snap-frozen for RNA analysis and the remaining tissue was collected for histological analysis.

2.5. Glucose Tolerance Test

Male mice were fasted overnight and basal glycaemia was measured from tail blood. Mice were administered a glucose solution of 2g/kg intraperitoneally, and glycaemia was measured at indicated timepoints using an automated glucose monitor. A minimum of 13 mice was used in each group.

2.6. Pancreatic Duct Ligation

A laparatomy was performed in 8-10-week-old wild type mice through a midline abdominal incision. The pancreatic ducts of the gastric and the splenic segments were ligated as described in Wang, *et al*., 1995. A double ligature was performed around the pancreas, just next to the blood vessels, in a region close to the great curvature of the stomach marked by the cranial mesenteric lymph nodes. The ligation totally obstructed exocrine flow from the gastrosplenic portion of the pancreas, which accounts for 50-60% of the entire pancreas.

3. EX VIVO PROCEDURES

3.1. Acinar cell isolation

Mice were sacrificed by cervical dislocation, and the skin and peritoneum were excised to expose viscera. A solution of Collagenase P (Roche) in HBSS at 1.33mg/ml was injected into different sites of the pancreas. After removal and placement in Collagenase P solution, the pancreas was cut into small pieces, and digested at 37 ºC for 20min in a shaking water bath. The resulting cell suspension was washed with HBSS 5% FBS, filtered through 105µm mesh to get

rid of islets and non-digested tissue. Finally, isolated acinar cells were placed on top of an HBSS 30% FBS solution and centrifuged to get rid of dead cells. Acinar cells were cultured in all cases in the following complete medium: RPMI supplemented with 1x sodium pyruvate, 1x Penicillin/Streptomycin, and Soybean Trypsin Inhibitor (STI) at 0.1mg/ml, and FBS at different concentrations depending on the experiment.

3.2. Acinar culture for RNA and IHC analysis

Isolated acinar cells were resuspended in complete medium with 10% FBS and cultured in suspension in anti-adherent Petri dishes at 37 ºC, 5% CO_2. After 48h, cells were collected, centrifuged, and the pellet was resuspended in fresh medium. Cells were cultured in the same conditions for an additional 48h.

For RNA extraction, an aliquot of cell suspension was taken 24h after seeding cells, washed with PBS, and RNA was isolated using Mammalian Total RNA Miniprep Kit (Sigma) according to manufacturer's indications.
For IHC analysis, freshly isolated acini and acinar cells cultured for 72h were taken, washed with PBS, fixed in PBS-buffered 4% formalin for 2h and resuspended in 2% low melting point Agarose. Acini embedded in agarose were included in paraffin to make blocks. Sections of 3µm were cut and immunohistochemical stainings were performed as described in section 4.2.

3.3. Medium conditioned by acinar cells and macrophage treatment

Isolated acinar cells were resuspended in RPMI supplemented with 1% FBS for 48h. The medium was collected, filtered through 45µm filters to remove debris and stored at -80 ºC.

Immortalized RAW264.7 macrophages were cultured in DMEM supplemented with 10% FBS until reaching 50-60% confluence. Then, medium was removed and medium conditioned by acini was added for 24h. Finally, cells were lysed and RNA was isolated using Mammalian Total RNA Miniprep Kit (Sigma) according to manufacturer's indications.

3.4. *In vitro* ADM induction

Isolated acinar cells were resuspended in complete medium supplemented with 1% FBS and growth factor-reduced matrigel at proportion 1:1. Acini were plated at appropriate concentration in wells of a 96-well plate previously coated with a thin layer of matrigel. After polymerization of matrigel-acini suspension, medium containing the indicated factors was added at the indicated concentrations: EGF (50ng/ml), IL17 (50ng/ml), IL6 (50ng/ml), IL1b (50ng/ml), IL2 (50ng/ml), IL4 (50ng/ml), IL10 (2ng/ml), Reg3b (500nM), Ccl5 (50ng/ml), Cxcl12

(50ng/ml). Medium was changed after 48h culture (day 3), and 48h later (day 5) number of cysts/well was quantified under the microscope.

4. HISTOLOGICAL ANALYSIS

4.1. Tissue preparation and histopathological analysis

Mouse pancreata were placed in 4% PBS-buffered formaldehyde and fixed overnight. After fixation, they were embedded in paraffin and serially sectioned (3μm). Sections from at least 3 representative areas of the whole tissue (150-200μm separation in thickness) were deparaffinized, stained with hematoxylin-eosin (H-E) and subjected to histopathological analysis. Evaluation of all carcinogenesis experiments was performed by Dr. Irene Espósito (Institute of Pathology, Heinrich-Heine-University, Düsseldorf). Quantification of the area affected by ADM/PanIN or by lipomatosis (and its degree) was assessed through visual inspection of pancreas sections and not through computerized analysis. Evaluation of the pancreatitis experiments in *Gata4*$^{P-/-}$ and control mice was performed by Dr. Mar Iglesias (Departament de Patologia, Hospital del Mar, Barcelona).

In order to quantify the area affected by PanIN and ADM in i-*Kras*;Gata4*$^{P-/-}$ and control mice induced with a chronic pancreatitis for 2 months, sections were digitally scanned (Mirax Scan, Zeiss) and processed according to pixel intensity. We took advantage of the fact that areas of PanIN and ADM have lumina that appear light-coloured in the H-E histology. We therefore trained the analysis software (AxioVision 4.6 software package, Zeiss) to distinguish areas of tissue including light-coloured pixels (damaged) from areas presenting darker pixels (not damaged). The software quantified the two types of areas and the total area, so that we were able to calculate percentage of damaged tissue.

Images from stained tissue sections were acquired with a Nikon Eclipse Ti microscope and managed with NIS-Elements BR3.2 software.

4.2 Immunohistochemical analysis

Paraffin-embedded tissue sections were deparaffinized in xylol, rehydrated with solutions of absolute, 96% and 70% EtOH, and distilled water. Sections were then boiled in 10mM sodium citrate buffer (pH 6.0) for 10 min to retrieve the antigens. Next, sections were incubated for 30 min with 3% H_2O_2 in methanol to block endogenous peroxidase and permeabilize cell membrane. Sections were washed with distilled water and blocked for 30 min with 2% BSA in PBS at room temperature. After blocking, the sections were incubated with primary antibodies, diluted in PBS with 2% BSA, for 1h at room temperature or overnight

at 4ºC. Next, sections were washed in PBS three times for 10min and incubated for 45min with EnVision+ HRP labeled secondary anti-rabbit antibodies (Dako). After this, sections were washed again and the signal was obtained using DAB+ Chromogen system (Dako). Finally, sections were rinsed with water, counterstained for 2min with Carazzi´s Hematoxylin solution DC (Panreac), dehydrated with increasing concentrations of alcohol, then with Xylol, and mounted using DePeX mounting medium (Gurr). The primary antibodies used are listed in Table M2.

Images from stained tissue sections were acquired with a Nikon Eclipse Ti microscope and managed with NIS-Elements BR3.2 software. Quantification of Mcm4+ and Ki67+ cells in control and Gata4$^{-/-}$ pancreata was performed by counting the proportion of positive cells in 5 random fields in the pancreas of ≥4 mice/genotype.

Antibody	Company, reference	Dilution
Goat α-Gata4	Santa Cruz, SC-1237	1:1000
Rabbit α-Ki67	Novocastra, NCL-Ki67p	1:1000
Rabbit α-Mcm4	Polyclonal sera provided by Dr. Juan Méndez, CNIO	1:400
Rat α-Krt19	Developmental Studies Hybridoma Bank, Troma III	1:1000
Rabbit α-cleaved Caspase-3	Cell Signaling, 9661	1:1000
Mouse α-gH2Ax Ser139	Upstate, 05-636,	1:2000
Rabbit α-p65	Santa Cruz, SC-372	1:500
Rabbit α-phospho-Stat3 Tyr705	Cell Signaling, 9145	1:200
Rabbit α-P53	Leica Byosistems NCL-L-p53-CM5p	1:500
Rabbit α-phospho-Gata4 Ser105	Abcam, ab5245	1:100
Mouse α-GFP (also for eYFP)	Roche, 11814460001	1:250
Goat α-Hnf1b	Santa Cruz, SC-7411	1:500
Rabbit α-Sox9	Millipore, AB5535	1:500
Mouse α-Glucagon	Sigma, G2654	1:500
Rat α-F4/80	ABD Serotec, MCA497 clone CI:A3-1	*
Rabbit α-Myeloperoxidase	Dako, A0398	*
Goat α-Cd3e	Santa Cruz, SC-1127 M-20	*
Goat α-Pax5	Santa Cruz, SC-1974 C-20	*
Rabbit α-Reg3b	Provided by Dr. Juan Iovanna, CRCM	1:400
Guinea pig anti-Insulin	Dako, A0564	1:500

Table M2. **Antibodies used in immunohistochemical analyses.** For some antibodies, the dilution is indicated with (*) as it was performed in CNIO histopathology unit.

Comentario [M1]: Revisando los anticuerpos que mencionas en resultados faltan: Muc1, E-cad, YPF, Glucagon.
Muc1: tabla IF
E-cad: tabla IF
YFP: el mismo que GFP. indicado
Glucagon: OK, need to check in the notebook

4.3. Immunofluorescence analysis

Tissue sections were processed as described in the previous paragraph, but skipping the blocking step with hydrogen peroxide. After incubation with the primary antibody (Table 3), sections were washed with PBS and incubated with a fluorescently-labeled secondary antibody at 1:200 dilution (Table M3). Next, sections were washed with PBS twice and with distilled H2O once, and incubated with DAPI (0.5µg/ml in dH_2O). Finally, tissues were mounted using ProLong® Gold Antifade Reagent (Life Technologies). Images were acquired using a confocal microscope (TCS SP5, Leica).

1ary Ab (dilution)	Company, reference	2ary Ab, spectra	Company, reference
Rabbit anti-Muc1 CT-1 (1:100)	Provided by (Ref)	Goat anti-rabbit, Alexa Fluor 555	Thermofisher, A-21428
Mouse anti-E-cadherin (1:200)	BD Transduction laboratories, #610182	Goat anti-Mouse, Alexa Fluor 488	Thermofisher, A-11001

Table M3. Antibodies (Ab) used in immunofluorescence analysis. Ref: Pemberton L, *et al*. Antibodies to the cytoplasmic domain of the MUC1 mucin show conservation throughout mammals. Biochem Biophys Res Commun 1992;185:167–75

4.4. Alcian Blue + P.A.S. staining

Sections were deparaffinized as described in section 2.2 and placed in distilled water. Alcian blue/Periodic acid-Schiff/Hematoxylin stain was performed using an automated staining platform (ArtisanLink Pro, Dako). In this procedure, slides were incubated in Alcian Blue solution for 5 minutes at 35ºC, in Periodic acid-Schiff for 13 minutes at room temperature, and in Mayer hematoxylin for 1 minute at room temperature; washing the slides between each step. The acidic mucins and neutral mucins vary in color (blue and magenta). The colour of the resulting staining in mixtures of acidic and neutral mucins depends on the dominant entity, and will range from blue-purple to violet or mauve. Hematoxylin provides nuclear counterstain. Finally, the slides were dehydrated, cleared and mounted with a permanent mounting medium for microscopic evaluation.

4.5. Evaluation of human TMA

A series of 23 evaluable cases was analysed. Only cases presenting one core with areas of normal pancreas with strong positivity in acinar cells were considered valid. A total of 18 different cases of normal pancreas as well as 27 low-grade PanIN, 2 high-grade PanIN, and 20 PDAC were scored for staining intensity.

Comentario [PR2]: source fo samples, informed consent, study, all that... Aquesta info no sé molt bé d'on treure-la, ho veiem junts

To determine histoscore (HS), we assigned a value for the intensity of staining, ranging from 0 (null) to 3 (strong). If the lesion was heterogeneous, we estimated the proportion of reactive cells showing a given intensity. We then multiplied percentage of cells to degree of staining and obtained a value ranging from 0-300. For lesions in different cores belonging to the same case, we did the average of the HS. We then classified HS values as follows: negative (0-49), low (50-99), intermediate (100-199), high (200-300).

5. RNA ANALYSIS

5.1. RNA isolation

Pieces of mouse pancreas were homogenized using T10 basic ULTRA-TURRAX homogenizer (IKA) in GTC buffer (4M Guanidine thiocyanate, 0.1M Tris-HCl, 1% 2-Mercaptoethanol, pH 7.5, prepared in DEPC treated water) and total RNA was extracted using Phenol-Chloroform method (Chirgwin *et al*., 1979).

5.2. Reverse transcriptase and quantitative PCR (RT-qPCR)

RNA was treated with DNase (DNA-free™ DNase Treatment & Removal Reagents, Ambion). mRNA was retro-transcribed with TaqMan® Reverse Transcription Reagents kit (Invitrogen). Quantitative PCR was performed using SYBR® Green PCR Master Mix in the 7500 Fast Real-Time PCR System for PCRs<96 samples and the QuantStudio 6 Flex system for PCRs >96 samples (both reagents and PCR machines from Thermofisher). mRNA expression was normalized with Hprt levels and calculated as fold-change over control samples. Primers used in this study are listed in Table M4.

Transcript	Forward primer	Reverse primer
Hprt	GGCCAGACTTTGTTGGATTTG	TGCGCTCATCTTAGGCTTTGT
Ptf1a	ACAAGCCGCTAATGTGCGAGA	TTGGAGAGGCGCTTTTCGT
Mist1	CCTTCAACTCTCCAGGGAAA	GCCACCACACATGCAATT
Cpa1	TACACCCACAAAACGAATCGC	GCCACGGTAAGTTTCTTGAGCA
Amy2a5	TGGCGTCAAATCAGGAACATG	AAAGTGGCTGACAAAGCCCAG
Pnlip	ACAGATCAACACCCGCTTTC	CGGGTTTTTCTGTTTGTTCG
Cela1	TGTGTCACACCCCTACTGGA	TTGTTAGCCAGGATGGTTCC
Cel	AAGTTGCCCGTGAAAAAGCAG	ATGGTAGCAAATAGGTGGCCG
Pdx1	AAATCCACCAAAGCTCACGC	CGGTCAAGTTCAACATCACTGC
Hnf1a	TAATAGGGCGGAGTGCAT	GGTCCGTTATAGGTGTCCAT
Gata6	GGTGCTCCACAGCTTACAGG	CACAGTGGCGTCTGGATG
Nr5a2	ATGTCCTGTGTGTGGCGATA	TCTGCGTTTTGTCAATTTGG
Cd3e	CGTCTACTGCTTTGCAGGACAT	CGATCTCGAAGAGGCTGATACA
Ccl5	GCCTCACCATATGGCTCGGACA	CCTTGACGTGGGCACGAGGC
Ccl19	GAAAGCCTTCCGCTACCTTC	CTGGTCTGGAGGTGCACAG
Icam1	CGCTGTGCTTTGAGAACTGTG	ATACACGGTGATGGTAGCGGA
Cxcl12	TGCATCAGTGACGGTAAACCA	CACAGTTTGGAGTGTTGAGGAT
Cxcr4	TGGAACCGATCAGTGTGAGT	GGTGGGCAGGAAGATCCTAT
Reg3b	GGCTTCATTCTTGTCCTCCA	AGATGGGTTCCTCTCCCAGT
Cxcl10	GCTGCCGTCATTTTCTGC	TCTCACTGGCCCGTCATC
Cxcl11	GGCTGCGACAAAGTTGAAGTGA	TCCTGGCACAGAGTTCTTATTGGAG
Nfkb	TGTCTGCTGCTGCTGGTGGC	CCACGTGGGCATCACCCTCC
RelA	TCTGGCGAATGGCTTTACTT	CTCCACATAAGGCCCAGAAG

Mmp9	GCAGAGGCATACTTGTACCG	TGATGTTATGATGGTCCCACTTG
Cxcl16	TTGGCACGGATCAGCGCCTTC	CCATCGCCTGGCAGGGTCAGC

Table M4. Sequences of primers used to detect the specified target genes mRNA. All sequences are expressed as 5'-3'.

5.3. RNAseq of mouse pancreata

<u>*Kras** and *Kras*;Gata4*$^{P-/-}$ mice</u>

Pancreata from 6 *Kras** and 6 *Kras*;Gata4*$^{P-/-}$ mice were collected and flash-frozen in liquid nitrogen. Tissue was mashed using a lab mortar and RNA from a portion of the whole tissue was extracted as described in section 4.1. Quality of RNA was assessed by determining RNA Integrity Number (RIN), and the 3 samples with higher values (above 8) were sent for library generation and next generation sequencing. Total number of reads/sample ranged from 33 to 40 million reads.

<u>Control and *Gata4*$^{P-/-}$ mice</u>

A piece of pancreas from a number of control (*Ptf1a*$^{+/Cre}$) and *Gata4*$^{P-/-}$ mice was processed and RNA was extracted following the protocol described in section 4.1. RIN was determined and samples with highest values (above 8) were mixed to generate pools of 3 different pancreata. A total of 3 pools were sent for library generation and deep sequencing. Total number of reads/sample ranged from 35 to 39 million reads.

In order to process the data we used Nextpresso v1.4 (Graña, *et al*., 2016), which allowed us to evaluate read quality, align reads to a mouse reference genome, and process the alignment to finally obtain a list of Diferentially Expressed Genes (DEG) between samples. Lists of DEG were then ordered according to the value of the test statistic used to compute significance of the observed change in FPKM (test_stat column in output file) and subjected to GSEA using KEGG library and an acinar-specific signature extracted from Masui *et al*., 2010 (Table M5). Statistical significance was set at False Discovery Rate (FDR)<0.05.

Acinar-specific gene signature							
Cpa1	Ctrb	Rnase1	Ela1	Pnliprp2	Prss3	Cpb1	Ela3b
Amy2a1	Cpa2	Reg1	Pnliprp1	Ela2a	Prss2	Ctrl1	
Klk1	Clps	Pnlip	Cel	Try4	Prss1	Ctrc	

Table M5. Digestive enzyme signature extracted from Masui *et al*., 2010.

5.4. Analysis of transcriptomic data from Bailey, et al. 2016

Data was extracted from Bailey, *et al*. 2016 supplementary data (GEO accession number GSE36924). Cases were stratified according to *GATA4* expression, and the ones above the median value were considered GATA4-high, and conversely, the ones under the median value were considered GATA4-low. Kaplan-Meier plots were performed according to the survival of each patient. The same procedure was applied to analyse the cases according to both *GATA4* and *GATA6* expression

For the GSEA analysis, gene expression data of cases on the top and bottom quartile according to *GATA4* expression was extracted (using the Comparative Markers Selection tool from Genepattern). Pre-ranked GSEA analysis was run on the list of the indicated genes.

6. CHROMATIN IMMUNOPRECIPITATION + QUANTITATIVE PCR (ChIP-qPCR)

Pancreas from wild-type mice was collected and cut into small pieces. Chromatin was cross-linked to proteins with 1% formaldehyde for 10min at room temperature. Fixation was stopped by adding glycine (to 0.125 M) for 5min. Next, tissue pieces were homogenized in ChIP SDS buffer (100 mM NaCl, 50 mM Tris [pH 8], 5 mM EDTA pH 8, 1% SDS) using a Dounce homogenizer. Lysates were sonicated at 8 ºC for 30min using a Covaris S2 Focused Ultrasonicator, (conditions: 10 Intensity, 10 duty cycle, 200 cycles/burst) yelding DNA fragments of 300-500bp. Lysates were diluted in Triton dilution buffer (100 mM Tris [pH=8.6], 100 mM NaCl, 5 mM EDTA, 5% Triton X-100) to reduce SDS concentration and allow protein-protein interactions. Chromatin in the lysates was pre-cleared by incubating with A/G beads and non-specific IgG for 2h at 4 ºC. After removing beads from pre-clearing, specific antibodies or non-related IgG, together with A/G beads, were added for immunoprecipitation of complexes overnight at 4 ºC. For each immunoprecipitation, 1ug of antibody was used (Goat anti-Gata4 SC-1237 (Santa Cruz); and same amount of goat IgG were used as non-related antibody. Next, beads were washed with Triton dilution buffer, mixed micelle buffer (150mM NaCl, 20mM Tris, 5 mM EDTA, 5% sucrose, 1% Triton X-100 and 0.2% SDS), buffer 500 (0.1% deoxycholic acid, 1mM EDTA, 50mM HEPES, 1% Triton X-100 and 500 mM NaCl), LiCl buffer (0.5% deoxycholic acid, 1mM EDTA, 250mM LiCl, 0.5% NP-40, 10mM Tris) and Tris-EDTA (TE). DNA was eluted in elution buffer (0.1M $NaHCO_3$, 1% SDS) and crosslinks were reversed by incubating overnight at 65ºC. RNA and protein were digested using RNase A and Proteinase K and DNA was purified by phenol–chloroform extraction and ethanol precipitation. Abundance of target DNA in material

Comentario [M3]: you can include sonication conditions (duty cycle,etc). Done!

subject to ChIP was assessed by qPCR using the following primers (sequences are written 5' to 3'): Reg3b Fw: GGTAGTGCTTGGCACAGGTT; Reg3b Rv: AGCAGGAGACTGCCTGTGAT; ChIP Negative Fw: TTGGGTGTTGGGAACTGAAT; ChIP Negative Rv: CCCTTCTCTGCCTTCTGATG.

7. MULTIPLE SEQUENCE ANALYSIS

Analysis of protein sequences to determine the relevance of Serine 105 of Gata4 was performed using the online tool T-COFFEE with the default settings (available at http://tcoffee.crg.cat/apps/tcoffee/do:regular). Protein sequences of all murine Gata members and Gata4 sequences from different species were obtained from the genome browser Ensembl (http://www.ensembl.org/index.html)

8. STATISTICAL ANALYSIS

Statistical analyses of quantitative variables were performed using Mann-Whitney test in experiments where $n<5$ and data did not follow a normal distribution, and two-tailed Student's test when $n\geq5$. Data are provided as mean±SEM. and $P<0.05$ was considered significant. Statistical analyses were performed using the software GraphPad PRISM version 6.0e.

Statistics for qualitative variables (presence/absence of PanIN, PDAC, etc.; degree of lipomatosis; degree of affectation in pancreatitis) was performed using Chi square test except for cases where frequencies<5, that we used Fisher's exact test. $P<0.05$ was considered significant. Statistical analyses were performed using the software GraphPad PRISM version 6.0e.

Statistical analysis of RNAseq data was performed by nextpresso v1.4 software. Significance threshold was set at $P<0.05$ after correcting for multiple testing.

RESULTS

1. GATA4 IN PANCREAS PHYSIOLOGY AND RESPONSE TO DAMAGE

1.1. *Gata4* inactivation in the pancreas does not lead to major alterations in exocrine pancreas architecture or function

GATA4 and GATA6 are the main GATA factors expressed in the normal human pancreas (Fig. 1A). To analyse the role of Gata4 in murine pancreatic development/homeostasis and compare it to the role of Gata6, we used a mouse strain in which exon 3, 4 and 5 of *Gata4* are floxed by two LoxP sites and are deleted upon Cre recombinase-mediated (Cre) inactivation. Exon 3 and 4 contain the Zn finger domains that are necessary for Gata4 binding to DNA, and its deletion results in the generation of a null allele (Fig. 1B). In order to achieve pancreas-specific deletion we have used a strain in which Cre is expressed under the control of *Ptf1a* (*Ptf1a*-Cre) at Embrionic stage (E)9-E9.5 in multipotent pancreatic progenitors, therefore resulting in *Gata4* deletion in all cells of the adult pancreas (Fig. 1B). This model will hereafter be referred to as *Gata4*$^{P-/-}$ mouse (P stands for pancreas-specific); the pancreas from these mice will be designated as *Gata4*$^{-/-}$ pancreas. As control for these mice, we used *Ptf1a*-Cre$^{+/KI}$ mice in all experiments.

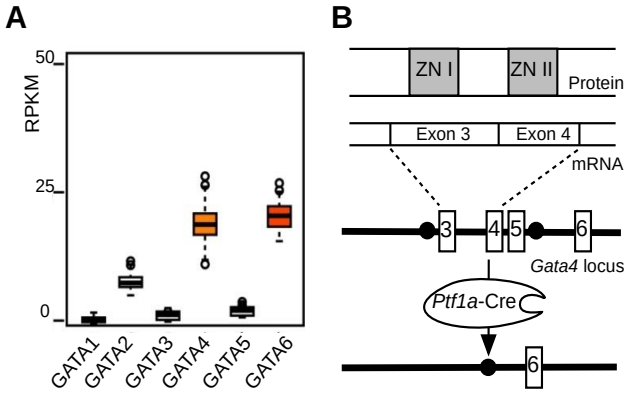

Figure 1. Gata4 is expressed in the pancreas at high levels as well as Gata6. (A) mRNA expression of GATA1-6 in normal human pancreas, showing that GATA4 and GATA6 are the GATA factors expressed at highest levels. Data was extracted from www.GTExportal.org. **(B)** Schematic representation of the strategy used to delete Gata4 in the mouse pancreas. The *Gata4* locus contains 2 loxP sites (black circles) flanking exons 3, 4 and 5 (numbered boxes), which contain the Zn finger domains through which Gata4 binds to DNA. Expression of Cre Recombinase under the promoter of *Ptf1a* results in the formation of a null allele.

We first assessed Gata4 expression in normal adult pancreas using immunohistochemistry (IHC): Gata4 is expressed in all acinar cells, but is undetectable in ductal and endocrine cells (Fig. 2A). The specificity of the antibody used was demonstrated using *Gata4*$^{-/-}$ pancreas, where we did not detect any reactivity, also indicating that recombination takes place effectively (Fig. 2A). Accordingly, wild-type (WT) Gata4 mRNA was undetectable in *Gata4*$^{-/-}$ pancreas (P=0.036) (Fig. 2B).

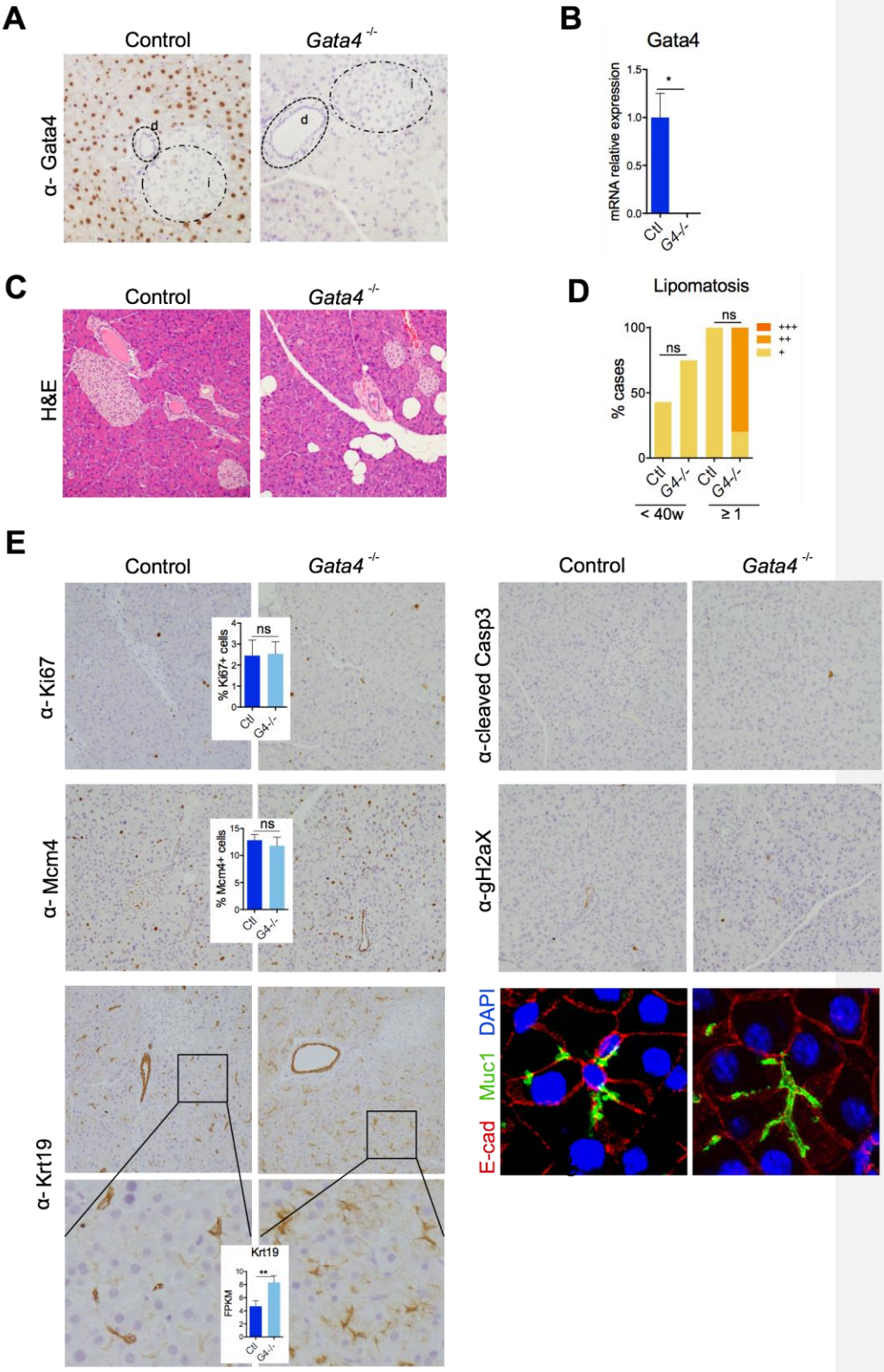
A
Control
Gata4 -/-
α- Gata4
d
i
B
Gata4
mRNA relative expression
1.5
1.0
0.5
0.0
*
Ctl
G4-/-
C
Control
Gata4 -/-
H&E
D
Lipomatosis
% cases
100
50
0
ns
ns
+++
++
+
Ctl
G4-/-
Ctl
G4-/-
< 40w
≥ 1
E
Control
Gata4 -/-
α- Ki67
% Ki67+ cells
ns
Ctl
G4-/-
α- Mcm4
% Mcm4+ cells
ns
α- Krt19
Krt19
FPKM
**
α-cleaved Casp3
α-gH2aX
E-cad
Muc1
DAPI

Figure 2. *Gata4* inactivation does not lead to major alterations in pancreas architecture or function. (A) IHC showing that Gata4 is expressed in acinar but not in endocrine or ductal cells (left panel). Upon recombination, Gata4 expression is efficiently lost in acinar cells in *Gata4*$^{P-/-}$ mice. **(B)** Normalized mRNA expression of Gata4 mRNA in control (Ctl, n=3) and *Gata4*$^{P-/-}$ mice (*G4*$^{-/-}$, n=5) assessed by RT-qPCR (P=0.0357). P>0.05 (ns); P<0.05 (*); P<0.01 (**); P<0.001 (***). **(C)** H&E staining showing that the pancreas of *Gata4*$^{P-/-}$ mice is histologically similar to control mice except for a mild increase in lipomatosis and a weaker eosinophilic staining. **(D)** Histopathological quantification of lipomatosis in *Gata4*$^{-/-}$ pancreas showing a non-statistically significant increase both at <40weeks (P=0.315) and >1 year (P=0.143). **(E)** Representative images and quantification of IHC stainings for markers of proliferation (Ki67) and DNA replication machinery (Mcm4), showing no significant differences. IHC stainings for DNA damage (gH2aX), apoptosis (cleaved-Caspase 3) and cell polarity (E-cadherin, Muc1) show no differences between *Gata4*$^{P-/-}$ and control mice. Expression of the ductal marker Krt19 is increased in *Gata4*$^{-/-}$ pancreas, both at protein (IHC) and mRNA level (RNA-seq data).

At the histological level, *Gata4*$^{-/-}$ pancreata of 30-week-old mice did not reveal any major abnormalities (Fig. 2C), except for a slightly weaker eosinophilic cytoplasm in acinar cells (Fig. 2C) and a non-statistically significant increase in lipomatosis both at early (<40 weeks) and late (≥1 year) timepoints (Fig. 2C, 2D). These findings might be indicative of a mild compromise in acinar function (Fig.2 C).

To further characterize the exocrine pancreas of *Gata4*$^{-/-}$ pancreata, we analysed the expression of markers of specific cell lineages, proliferation, apoptosis, DNA damage, and cell polarity. We found an increased expression of Krt19 in the cytoplasm of acinar cells, with no differences in expression in ductal cells, suggesting an incipient acinar-to-ductal metaplasia (ADM) (Fig. 2E). Accordingly, RNA-seq data of *Gata4*$^{-/-}$ and control pancreata (8 weeks) showed a significant increase of Krt19 levels (Fig. 2E). Muc1 and E-cadherin were detected in mutually exclusive cell membrane domains, indicating the maintenance of acinar cell polarity. We did not find significant differences in the proportion of Ki67 and Mcm4-positive cells in control and *Gata4*$^{-/-}$ pancreata (Fig. 2E). These results are unlike those found in 8-week-old *Gata6*$^{P-/-}$ mice where increased proliferation (Ki67, Mcm4), DNA damage (gH2aX), apoptosis (cleaved-Caspase3), and altered cell polarity (data not shown) were observed (Martinelli *et al*., 2012). Even more, pancreata of 30-week old *Gata4*$^{-/-}$ mice looked similar to controls except for a higher degree of lipomatosis (Fig. 2D), while *Gata6*$^{-/-}$ pancreata presented with extensive lipomatosis, acinar atrophy and acinar-to-ductal metaplasia (ADM) (Martinelli *et al*., 2012).

To identify subtle changes in cell differentiation, we performed transcriptomic analysis using RNA-seq from whole pancreas of control and *Gata4*$^{P-/-}$ mice (n=3 for each group). Principal Component Analysis (PCA) showed that the two groups clustered separately, indicating the existence of global transcriptomic differences (Fig. 3A). Visual inspection of the data revealed that the top 15 differentially expressed genes downregulated in *Gata4*$^{-/-}$ pancreata included a number of digestive enzymes such as Trypsin (*Try10, Prss3*), Lipase (*Lpl*) and RNAse (*Rnase1*); as well as some members of the Reg family genes (*Reg3a,*

Reg3b, Reg3g, Reg3d). By contrast, the list of top genes upregulated in *Gata4*$^{-/-}$ pancreata was very diverse, including a serine protease (*Klk1b4*), a neuroendocrine peptide (*Gal*) or angiotensinogen (*Atg*), which is typically expressed in the liver (Table 1).

We then performed Gene Set Enrichment Analysis (GSEA) comparing both transcriptomes in order to identify the pathways that were deregulated in each condition. We ran the analyses applying the KEGG library so that a set of gene signatures representing the majority of biological processes was assessed. We did not find any statistically significant upregulated pathways in *Gata4*$^{-/-}$ pancreata (Fig. 3B). We only found two pathways that were significantly downregulated in *Gata4*$^{-/-}$ pancreata (FDR<0.05), "PPAR signalling pathway" and "Hematopoietic cell lineage". In addition, "Complement and coagulation cascades" (FDR=0.08), "Systemic lupus erythematosus" (FDR=0.257) and "Primary immunodeficiency" (FDR=0.276) pathways appeared downregulated in *Gata4*$^{P-/-}$ pancreata albeit with a lower statistical significance (Fig. 3B). These results suggest that Gata4 might play a role in the regulation of inflammatory/immune processes in basal conditions. We also interrogated a customized signature that includes an acinar geneset containing genes coding for digestive enzymes (extracted from Masui *et al*., 2010) (Fig. 3C). We found a significant downregulated of this signature in *Gata4*$^{-/-}$ pancreata (FDR=0.002), indicating a dysfunction in enzyme production upon Gata4 loss.

To validate these findings, we used RT-qPCR to analyse the expression of specific genes that are important for acinar cell function, including enzymes (*Pnlip, Amy2a5, Cpa1, Cela1, Cel*) and regulators of the acinar programme (*Mist1, Pdx1, Hnf1a, Ptf1a, Gata6, Nr5a2*) (Fig. 3D). According to the results obtained in the GSEA analysis applying the Masui *et al.* signature, we found that the majority of digestive enzymes checked by RT-qPCR (*Pnlip, Amy2a5, Cpa*) were significantly downregulated in *Gata4*$^{-/-}$ pancreata. However, we also observed enzymes with unaltered (*Cela1*) or even upregulated expression (*Cel*) in the *Gata4*$^{-/-}$ pancreas. Regarding the acinar-related transcription factors, there was also not a consistent pattern as some showed similar expression levels (*Mist1, Pdx1, Hnf1a*) but others were upregulated in the *Gata4*$^{-/-}$ pancreas (*Ptf1a, Gata6, Nr5a2*). Similar results were found in the RNA-seq data for these genes (data not shown). These results are unlike those found in *Gata6*$^{-/-}$ pancreata, where a more severe, consistent and uniformly reduced expression of acinar genes and their transcriptional regulators was observed (Martinelli *et al*., 2012).

Altogether, we conclude that Gata4 is largely dispensable for the development and differentiation of the exocrine pancreas after E9.5 and that Gata6 plays a

Upregulated in *Gata4*-/- pancreas

Gene name	test_stat	q-value	Gene product	Description
Klk1b4	11,539	0,0011	Kallikrein 1	Serine protease, involved in generating plasmin from plasminogen
Gal	10,638	0,0011	Galanin; GMAP Prepropeptide	Neuroendocrine precursor that generates Galanin (feeding and energy homeostasis) and GMAP (antifungal)
Agt	10,523	0,0011	Angiotensinogen	Expressed in the liver, it is cleaved by the enzyme Renin in response to lowered blood pressure
Rbp4	10,415	0,0011	Retinol Binding Protein 4	Specific carrier that delivers retinol from the liver stores to the peripheral tissues
Kng2	9,902	0,0011	Kininogen 1	HMWK (generated by alternative splicing): role in blood coagulation and assembly of the kallikrein-kinin system
Bdh2	8,691	0,0011	3-Hydroxybutyrate Dehydrogenase	Related to Butanoate metabolism
Klk1b24	8,587	0,0011	Kallikrein 1	Serine protease, involved in generating plasmin from plasminogen
Enho	8,082	0,0011	Energy Homeostasis Associated	Involved in the regulation of glucose homeostasis and lipid metabolism
Gc	7,946	0,0011	GC, Vitamin D Binding Protein	Involved in VitD transport and storage, it enhances the chemotactic activity of C5a for neutrophils and macrophages
Dct	7,828	0,0011	Dopachrome Tautomerase	Involved in regulating eumelanin and phaeomelanin levels
F2	7,608	0,0011	Coagulation Factor II, Thrombin	Coagulation factor II is proteolytically cleaved to form thrombin in the first step of the coagulation cascade
Ppp1r1b	7,286	0,0011	Protein Phosphatase 1 Regulatory Inhibitor 1B	Diseases associated with PPP1R1B include Schizophrenia and Attention Deficit-Hyperactivity Disorder
Foxc1	6,876	0,0011	Forkhead Box C1	Involved in the regulation of embryonic and ocular development
Cdo1	6,860	0,0011	Cysteine Dioxygenase Type 1	Initiates metabolic pathways related to pyruvate and sulfurated compounds. Critical regulator of cellular cysteine
Serpina10	6,643	0,0011	Serpina10	Expressed in the liver and secreted in plasma, it inhibits the activity of coagulation factors Xa and Xia

Dowregulated in *Gata4*-/- pancreas

Gene name	test_stat	q-value	Gene product	Description
Gdf9	-nan	0,0011	Growth Differentiation Factor 9	Secreted ligand of the TGF-beta superfamily
Hsd17b13	-15,122	0,0011	Hydroxysteroid 17-Beta Dehydrogenase 13	Enzyme with oxidoreductase activity related to lipoprotein metabolism
Reg3a	-14,991	0,0011	Regenerating Family Member 3 Alpha	Pancreatic secretory protein that involved in cell proliferation and/or differentiation
Reg3b	-14,599	0,0011	Regenerating Family Member 3 Beta	Pancreatic secretory protein that involved in cell proliferation and/or differentiation
Try10	-13,471	0,0011	Trypsinogen	Pancreatic digestive enzyme
Prss3	-12,938	0,0011	Trypsinogen	Pancreatic digestive enzyme
Reg3g	-12,871	0,0011	Regenerating Family Member 3 Gamma	Pancreatic secretory protein that may be involved in cell proliferation and/or differentiation
Hmgcs2	-11,564	0,0011	3-Hydroxy-3-Methylglutaryl-CoA Synthase 2	Catalyzes the first reaction of ketogenesis, which provides lipid-derived energy during carbohydrate deprivation
Sostdc1	-10,623	0,0011	Sclerostin Domain Containing 1	Bone morphogenetic protein (BMP) antagonist
Reg3d	-10,055	0,0011	Regenerating Family Member 3 Delta	Pancreatic secretory protein that involved in cell proliferation and/or differentiation
Lpl	-9,125	0,0011	Lipoprotein Lipase	Pancreatic digestive enzyme
Rnase1	-8,899	0,0011	Ribonuclease 1	Pancreatic digestive enzyme
Zbtb18	-8,720	0,0011	Zinc Finger And BTB Domain Containing 18	Zinc finger transcriptional repressor of genes involved in neuronal development
Gm5771	-8,631	0,0011	Trypsinogen	Pancreatic digestive enzyme
Ggh	-8,562	0,0011	Gamma-Glutamyl Hydrolase	Catalyzes the hydrolysis of folylpoly-gamma-glutamates and antifolylpoly-gamma-glutamates

Table 1. Top upregulated and downregulated genes in *Gata4*$^{-/-}$ pancreas. Test_stat: value of the test statistic used to compute significance of the observed change in FPKM (nan: infinitive due to FPKM=0 in one condition); q-value: normalized p-value; gene product: protein; and description of its function.

more prominent role in acinar differentiation. Of note, these results are opposite to what had been proposed in the literature in the last decade (Decker *et al*., 2006; Ketola *et al*., 2004; Ritz-Laser *et al*., 2005).

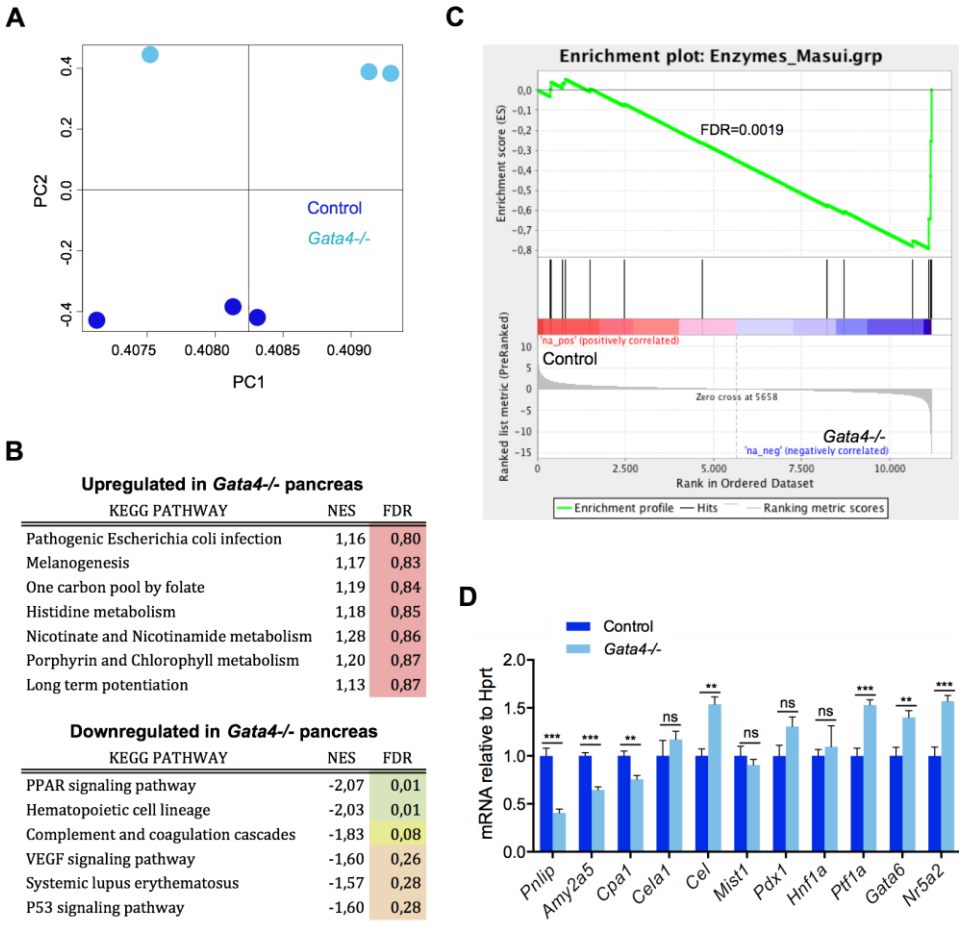

Figure 3. *Gata4* inactivation perturbs pancreatic gene expression. (A) Principal Component Analysis (PCA) of the RNA-Seq data from 3 control (dark blue) and 3 *Gata4*$^{-/-}$ (light blue) pancreata. Principal Component 2 (PC2) separates the two groups, evidencing the effect of Gata4 loss in pancreatic gene expression. **(B)** Gene Set Enrichment Analysis (GSEA) comparing the transcriptome of *Gata4*$^{-/-}$ and control pancreas. Significance threshold was set at FDR<0.05, although pathways with FDR<0.25 were considered of interest as well. The table shows the lack of significantly up-regulated pathways in *Gata4*$^{-/-}$ pancreata and the few pathways significantly down-regulated in *Gata4*$^{-/-}$ pancreas (NES: normalized enrichment score; FDR: False Discovery Rate). **(C)** GSEA showing positive enrichment (FDR=0.0019) of a signature from Masui, *et al*. that includes the main acinar genes whose expression depends on a functional PTF1 transcriptional complex. **(D)** Relative mRNA expression, assessed by RT-qPCR, of genes related to acinar function, including those coding for digestive enzymes (*Pnlip, Amy2a5, Cpa1, Cela1, Cel*) and transcription factors (*Mist1, Pdx1, Hnf1a, Ptf1a, Nr5a2, Gata6*). P>0.05 (ns); P<0.05 (*); P<0.01 (**); P<0.001 (***).

1.2. *Gata4* inactivation in the pancreas leads to a mild perturbation of glucose metabolism

We then characterized the role of Gata4 in the endocrine compartment. Gata4 was undetectable in the normal islets of Langerhans using IHC (Fig. 2A). However, fasting basal glycaemia was slightly lower in *Gata4*$^{P-/-}$ mice (P<0.001). Upon intraperitoneal glucose administration, *Gata4*$^{P-/-}$ mice showed lower glycaemia at all time points of analysis until 2h post-glucose administration (Fig. 4A). Immunostaining for glucagon and insulin did not reveal any clear-cut differences in intensity or distribution in both mouse strains (Fig. 4C).

We next analysed expression levels of endocrine regulators by interrogating the RNA-seq data for endocrine-related genes, including transcription factors (*Hnf4a, Neurod1, Mafa, Nkx6-2*) and hormones (*Gcg* and *Ins1*) and did not find any significant differences in expression (Fig. 3B). Altogether, these results suggest that lack of Gata4 has a mild impact on the regulation of glucose homeostasis; the analysis of the mechanisms involved therein was not pursued as it is beyond the scope of my aims. Further studies will be necessary to determine the mechanisms through which Gata4 is plays a role in endocrine pancreas homeostasis.

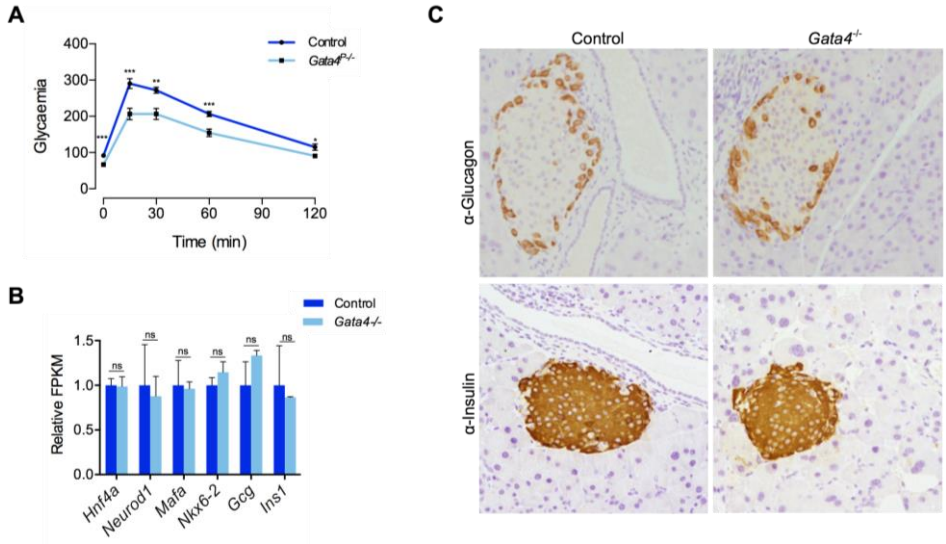

Figure 4. Analysis of endocrine gene expression and function in *Gata4*$^{P-/-}$ mice shows a mild alteration in glucose metabolism. (A) Glycaemia upon IP glucose administration. *Gata4*$^{P-/-}$ mice present a significantly lower basal glycaemia (after an overnight fasting, P<0.001) and maintain the same trend at 15 minutes (P<0.001), 30 minutes (P=0.0014), 60 minutes (P<0.001), and 120 minutes (P=0.018) post-injection. P>0.05 (ns); P<0.05 (*); P<0.01 (**); P<0.001 (***). **(B)** Expression of genes related to endocrine function, including transcription factors (*Hnf4a, Neurod1, Mafa, Nkx6.2*), and the hormones glucagon (Gcg) and insulin (Ins1), assessed by RNA-seq. **(C)** Representative images of IHC stainings of glucagon and insulin in control and *Gata4*$^{P-/-}$ mice showing no overt differences in intensity or distribution of the hormones within the islet of Langerhans.

1.3. Gata4 is phosphorylated in acinar cells upon stress

Gata4 is known to play an important role in cardiogenesis (Pu *et al*., 2004; Watt *et al*., 2004) and in the response to cardiac stress (Oka *et al*., 2006). Studies performed in this field have shed light on how Gata4-mediated transcriptional regulation is modulated by post-translational modifications. Specifically, Gata4 S105 phosphorylation has been reported to be required for optimal transcriptional activity during cardiac hypertrophy after tissue injury, suggesting that Gata4 is activated in response to stress (Van Berlo *et al*., 2011). The relevance of S105 phosphorylation of Gata4 was underlined by the fact that, among all Gata proteins, only Gata4 has a Serine at position 105, suggesting that phosphorylation at this residue contributes to Gata4-specific functions (Fig. 5A).

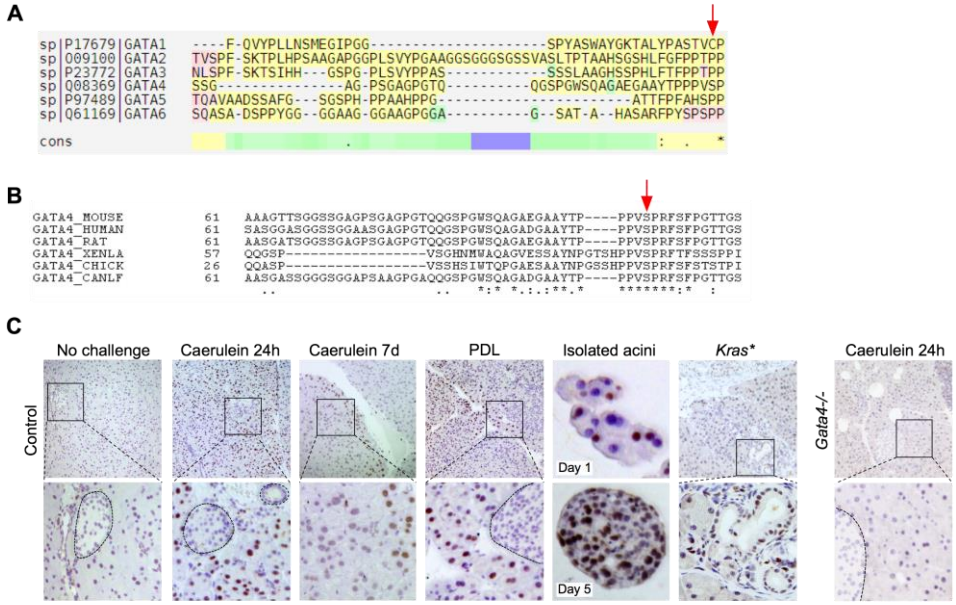

Figure 5. Gata4 is phosphorylated in acinar cells under stress conditions. (A) Alignment of the amino acid sequence of the 6 mouse Gata members using T-COFFEE multiple sequence alignment (MSA). Red arrow indicates the residue S105 that undergoes phosphorylation and is absent in other Gata members, supporting that it contributes to Gata4-specific functions. **(B)** Alignment of the amino acid sequence of Gata4 from mouse, human, rat, Xenopus, chicken and dog using MSA. Red arrow shows that the S105 residue is conserved across all analysed species, suggesting an important role of this residue for Gata4 function. **(C)** IHC analysis of phospho-S105-Gata4 in basal conditions and upon induction of pancreatic damage or challenge. Ducts and islets are delimited with a dashed line. From left to right, representative images (low and high magnification) of wild-type pancreas (unless specified) in the following conditions: **I.** Basal state (no challenge). **II.** Caerulein-induced acute pancreatitis (24 h time point, inflammatory stage). **III.** Caerulein-induced acute pancreatitis (7 day time point, recovery stage). **IV.** Obstructive pancreatitis induced by Pancreatic Duct Ligation (PDL), (5 days after surgery, inflammatory stage). **V.** Freshly isolated acinar cells, analysed immediately after isolation (upper image) or after culture for 5 days in suspension (lower). **VI.** Kras*-induced acinar-to-ductal metaplasia. **VII.** The specificity of the antibody used is demonstrated by the lack of immunostaining in the pancreas of *Gata4*$^{p-/-}$ mice 24h after the induction of acute caerulein pancreatitis.

In addition, we aligned Gata4 amino acid sequences of a number of species (mouse, human, rat, Xenopus, chicken and dog) and found that the residue S105 is conserved across species, supporting the idea that it plays an important role in Gata4 activity (Fig. 5B).

To determine whether Gata4 is phosphorylated in the pancreas in basal conditions or upon damage/stress, we stained for phospho-Gata4 at S105 (pGata4) in different conditions in which the pancreas is challenged (Fig. 5C). The specificity of the antibody used was assessed using samples from *Gata4*$^{-/-}$ mice 24h post-caerulein administration; no phospho-Gata4-expressing cells were identified (Fig. 5C).

Phospho-Gata4+ cells were nearly undetectable in basal conditions in all cellular compartments of normal pancreas. According to Gata4 expression pattern described previously, we did not find any cell from normal ducts or from the endocrine compartment positive for phospho-Gata4. However, upon induction of a caerulein acute pancreatitis, the majority of acinar cells stained for phospho-Gata4 at the 24h time point (inflammatory stage), while only residual acinar cells were phospho-Gata4+ at day 7 (recovery stage), suggesting a transient phosphorylation that is dissipated when tissue is recovered.

Similarly, we observed phopho-Gata4 staining in acinar cells in other models in which acinar cells undergo damage/stress, such as in obstructive pancreatitis induced by pancreatic duct ligation (PDL), in isolated acinar cells cultured in suspension –displaying an embryonic precursor phenotype that shares features with chronic pancreatitis (Pinho *et al*., 2011), and in Kras*-induced acinar-to-ductal metaplasia (Fig. 5C).

We conclude that Gata4 is phosphorylated in the pancreas upon stress conditions, which may be indicative of enhanced DNA binding and transcriptional activity as reported in other systems (Li *et al*., 2012). Therefore, Gata4 might play an important role in conditions where acinar cells respond to damage/stress rather than in basal conditions.

1.4. *Gata4*$^{P-/-}$ mice recover normally from acute pancreatitis

To determine whether the mild exocrine defect identified in *Gata4*$^{P-/-}$ mice compromises their response to damage, we induced a mild acute pancreatitis by administering 7 hourly injections of a supramaximal dose of caerulein to *Gata4*$^{P-/-}$ mice and controls (*Ptf1a*-Cre$^{+/KI}$ mice); we included additional control mice of both genotypes receiving saline. We analysed signs of damage and inflammation [edema, inflammatory cell infiltration, vacuolization, and acinar-ductal metaplasia (ADM)] during the inflammatory phase (24h and 48h after caerulein administration) and during the recovery phase (7 and 14 days after caerulein

administration) (Fig. 6A). *Gata4*$^{P-/-}$ mice presented with a slight increase in edema, acute inflammation and vacuolization, suggesting that the pancreatitis in a *Gata4*$^{-/-}$ context is slightly more severe, but the differences were not statistically significant. Two out of 6 *Gata4*$^{P-/-}$ mice presented moderate, focal, ADM at 48h, indicating that ADM occurs in the absence of Gata4 (Fig. 6B), a concept that will be important when discussing coming results. Altogether, these data suggest that while Gata4 may play a role during pancreatic regeneration upon acute pancreatitis, its absence does not compromise the ability of the tissue to recover.

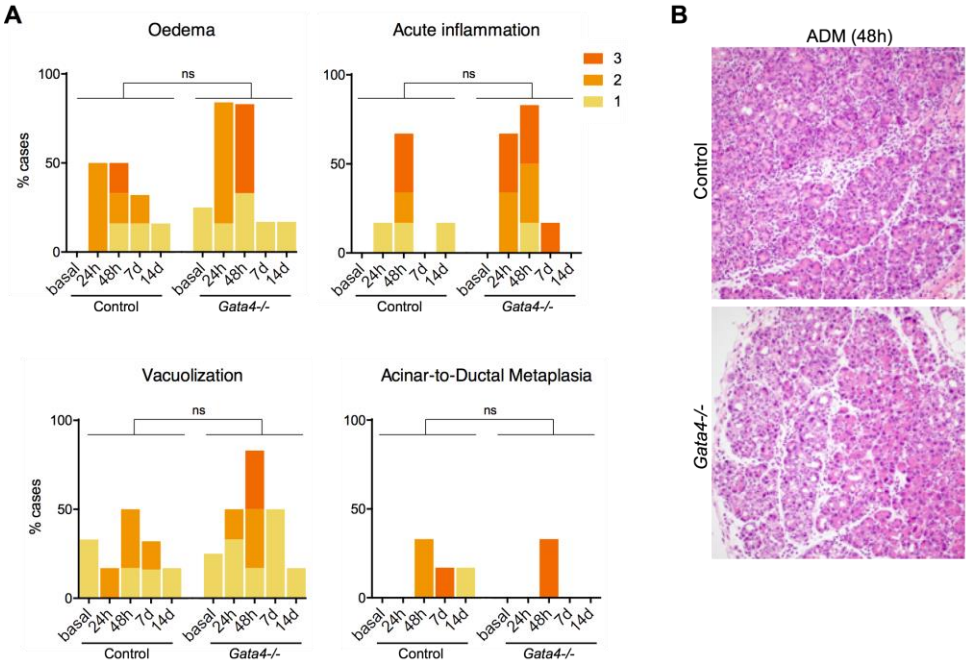

Figure 6. *Gata4*$^{P-/-}$ and control mice display a similar response to the induction of an acute pancreatitis. An acute pancreatitis was induced by 7 hourly injections of a supramaximal dose of Caerulein. Pancreata were harvested at 24h and 48h post-administration (inflammatory stage), and at day 7 and 14 post-administration (recovery stage). For each timepoint, 6 mice were analysed. Mice receiving a saline solution are included as basal state (n=3 for controls; n=4 for *Gata4*$^{P-/-}$). **(A)** Inflammation in the acinar parenchyma was scored according to the presence of oedema, leukocyte infiltration, acinar vacuolization, and ADM and categorized as: 1=minimum; 2=mild; 3=moderate. P>0.05 (ns); P<0.05 (*); P<0.01 (**); P<0.001 (***). **(B)** Representative images at 48h post-Caerulein of mild and moderate ADM in *Gata4*$^{P-/-}$ and control mice, respectively.

2. ROLE OF GATA4 IN MOUSE PANCREATIC CARCINOGENESIS

2.1. *Gata4*$^{P-/-}$ mice develop PDAC, but not PanIN, in mutant Kras-driven carcinogenesis

Given the prominent role of Gata6 as a tumour suppressor during pancreatic carcinogenesis, we asked whether Gata4 could also be involved in tumour initiation and progression in a model of mutant Kras-driven pancreatic tumourigenesis. For this purpose, we crossed *Gata4*$^{P-/-}$ mice with LSL-*KRas*G12V$^{+/KI}$ to generate *Ptf1a*-Cre$^{+/KI}$; *Gata4*$^{lox/lox}$; LSL-*KRas*G12V$^{+/KI}$ (termed *Kras*;Gata4*$^{P-/-}$). We analysed the histology of the pancreas at three different timepoints: in mice younger than 40 weeks (<40w), representing a pre-tumoural stage; in 1 year-old mice and in mice that were 1.5 years of age (1.5y), when many *Kras** mice have already developed tumours (Guerra *et al.*, 2007). We quantified the prevalence of known pre-neoplastic lesions such as Atypical Flat Lesions (AFL) and Pancreatic Intraetpithelial Neoplasia (PanIN), and Pancreatic Ductal Adenocarcinoma (PDAC), as well as the percentage of remodelled area. All histological analyses were performed blindly by an internationally recognized pathologist with extensive expertise in both human and mouse pancreatic pathology. At least 3 different areas of the tissue with a minimum of 150μm distance in thickness between them were analysed.

Pre-neoplastic lesions

We found that most *Kras** mice presented at least one focus of low-grade PanIN at <40w (5/7 cases), while none of the *Kras*;Gata4*$^{-/-}$ (0/10 cases) of the same age showed any PanINs (P=0.003). The differences were even more drastic at 1y and 1.5y, where all (14/14 and 14/14, respectively) *Kras** mice presented low-grade PanIN and only 1/23 *Kras*;Gata4*$^{-/-}$ cases at 1y (P<0.001) and 1/6 cases at 1.5y (P<0.001) presented one focus of PanIN. In addition to the number of cases presenting PanIN, we quantified the area of acinar tissue that had undergone remodelling, which includes formation of ADM and PanIN lesions (Fig. 7B), and found a dramatic decrease -or absence- in *Kras*;Gata4*$^{P-/-}$ mice at <40w (P=0.027), at ≥1y (P<0.001), and at 1.5y (P=0.009).

At <40w we found 2/7 *Kras** mice with AFL, and similarly to PanIN quantification, we did not observe any AFL in *Kras*;Gata4*$^{P-/-}$ (0/10 cases) (P=0.154). Similar results were observed at 1y, with 10/14 *Kras** mice presenting AFL and only 1/23 *Kras*;Gata4*$^{-/-}$ case presenting one focus of AFL (P<0.001). Similarly, at 1.5y we observed AFL in 7/14 *Kras** and in 1/6 *Kras*;Gata4*$^{P-/-}$ (P=0.325). Differences at <40w and 1.5y were not significant probably due to the lower number of cases analysed. Interestingly, the 1-year-old *Kras*;Gata4*$^{-/-}$ case presenting one focus of PanIN was the same mouse presenting an AFL, and both lesions were in the vicinity of an early carcinoma,

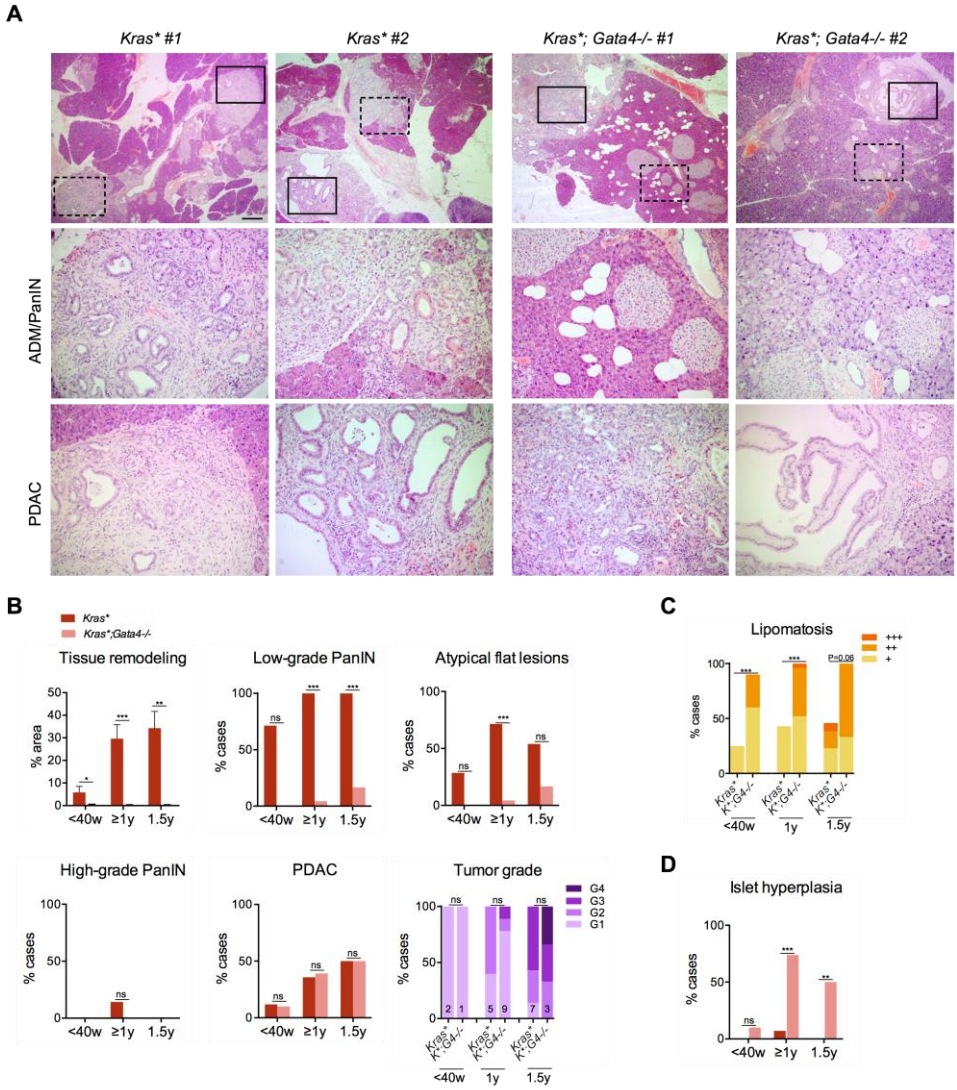

Figure 7. Gata4 is necessary for the development of PanIN lesions, but not for PDAC. (A) Representative histological findings in 2 *Kras** and 2 *Kras*;Gata4*$^{-/-}$ pancreata from 1-year-old mice. The low-magnification view shows representative areas of ADM/PanIN (dotted square), and areas of PDAC (normal square), which are magnified below. **(B)** Quantification of tissue remodelling and incidence of low-grade PanIN, Atypical Flat Lesions (AFL), high-grade PanIN and PDAC, including tumour grade. The analysis was stratified according to age (<40 weeks; 1 year; and 1.5 years). Tissue remodelling, presence of low-grade PanIN and AFL was almost absent in the pancreas of *Gata4*$^{P-/-}$. By contrast, the incidence of PDAC was similar in the two groups. P>0.05 (ns); P<0.05 (*); P<0.01 (**); P<0.001 (***). Number of *Kras** mice analysed: N(<40w)=10; N(1y)=14; N(1.5y)=14. Number of *Kras*;Gata4*$^{P-/-}$ mice analysed: N(<40w)=7; N(1y)=23; N(1.5y)=6. For tumour grade, only mice bearing tumours were analysed, and the number of cases is displayed within the bar. For *Kras** mice at <40weeks, PDAC incidence was analysed in an extended series of 17 cases. **(C)** Quantification of lipomatosis, which is significantly increased in *Kras*;Gata4*$^{-/-}$ pancreas both at <40w and 1y (P<0.001), and maintains the trend at 1.5y (P=0.06) **(D)** Quantification of islet hyperplasia incidence, which is significantly increased in Kras*;*Gata4*$^{-/-}$ pancreata at 1y (P<0.001) and at 1.5y (P<0.05).

therefore they might be part of the developing tumour and not isolated lesions. Accordingly, the 1.5-year-old *Kras*;Gata4*$^{P-/-}$ cases with either a PanIN or an AFL were both presenting PDAC as well.

High-grade PanIN lesions and PDAC

High-grade PanINs were absent in both *Kras** and *Kras*;Gata4*$^{P-/-}$ mice at <40w and 1.5y. For the largest timepoint dataset (1y) we observed 2/14 *Kras** cases presenting high-grade PanIN and 0/23 *Kras*;Gata4*$^{P-/-}$, this difference being statistically non-significant (P=0.137) (Fig. 7B). Low incidence of high-grade PanIN may be attributed to the fact that these lesions are frequently considered *in situ* carcinomas, therefore classified as PDAC. Strikingly, despite the lack of ADM, PanIN or AFL, tumours did develop in *Kras*;Gata4*$^{P-/-}$ mice. At <40w we found that 2/17 *Kras** and 1/10 *Kras*;Gata4*$^{P-/-}$ mice had developed tumours (P=1.00). At 1y of age 5/14 *Kras** and 9/23 *Kras*;Gata4*$^{P-/-}$ presented PDAC (P=1.00). Finally, at 1.5 years old we found 7/14 *Kras** with PDAC, and 3/6 *Kras*;Gata4*$^{-/-}$ with tumour (P=1.00) (Fig. 7B). Notably, the incidence of PDAC was not statistically different between the two groups, neither at <40w, 1y or 1.5y of age, indicating that tumours can arise in *Kras*;Gata4*$^{P-/-}$ mice at similar frequencies than *Kras** despite the absence of lesions typically described as tumour precursors. Analysis of tumour grading by the pathologist revealed no major differences between genotypes.

Additional histological findings

During the histopathological analyses, two more observations called our attention. The first was the high degree of lipomatosis in *Kras*;Gata4*$^{P-/-}$ mice (Fig. 7C), already present at <40w (P<0.001) and more prominent at 1y of age (P<0.001) and, although not significant due to few cases, also at 1.5y (P=0.06). The second observation was the presence of islet hyperplasia (Fig. 7D), specially in mice ≥1y of age, where 17/23 *Kras*;Gata4*$^{P-/-}$ mice presented hyperplasia compared to 1/14 *Kras** (P<0.001); and also at 1.5y of age, where 3/6 *Kras*;Gata4*$^{P-/-}$ mice presented prominent islets and none of the *Kras** did (P=0.004).

2.2. Acute pancreatitis does not promote PanIN development in *Gata4*$^{P-/-}$ mice

Several groups, including ours, have reported that a single episode of acute pancreatitis is able to increase PanIN incidence in mice carrying a mutant *Kras* allele (Carrière, *et al*., 2009, 2011; Flandez *et al*., 2014). To determine whether *Kras*;Gata4*$^{P-/-}$ mice are able to develop PanIN lesions, we induced a two-day caerulein acute pancreatitis consisting in 7 hourly injections of supramaximal dose of cerulein the first day, one day intermission, and repeated the same

procedure the following day. We treated two cohorts of mice at "early" and "late" timepoints, 20 and 40 weeks respectively, and histological analyses were performed at age 1 year. The frequency of low-grade PanIN, high-grade PanIN, AFL, PDAC, tumour grading, lipomatosis and islet hyperplasia were assessed as described in previous sections in "early" and (Fig. 8A) and "late" mice (Fig. 8B). As expected, we observed that all "early" and "late" *Kras** mice presented low-grade PanINs, several of them with many foci of lesions (more than 20 foci). In agreement with other reports, we found an increase in the proportion of mice presenting PDAC, from 35.71% (5/14) in non-challenged mice (Fig. 7B) to 75% (3/4) in "early" and 57.14% (4/7) in "late" mice (Fig. 8A,B), indicating that pancreatitis is a tumour-accelerating event. Regarding AFL incidence, we found no differences comparing non-challenged mice (71.43% cases; 10/14) (Fig. 7B) to mice induced with pancreatitis at "early" (75% cases; 3/4) or "late" (85.71% cases; 6/7) timepoints (Fig. 8A, 8B).

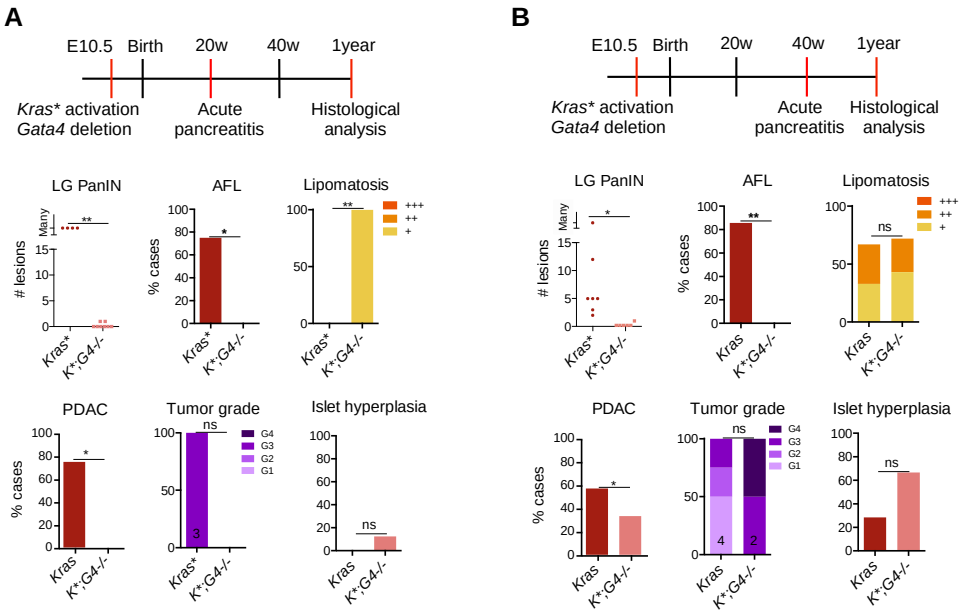

Figure 8. Acute pancreatitis does not result in increased PanIN formation in *Kras*;Gata4*$^{-/-}$ pancreas. (A) Top: experimental design. An acute pancreatitis was induced in 20 week-old *Kras** and *Kras*;Gata4*$^{P-/-}$ (*K*;G4*$^{-/-}$) mice; analysis was performed at 1 year of age. Bottom: Histopathological scoring of low-grade PanIN (LG PanIN), AFL, PDAC and its grading, lipomatosis and islet hyperplasia (n=5 for *Kras*;* n=7 for *Kras*;Gata4*$^{P-/-}$). For tumour grade, only mice bearing tumours were analysed, and the number of cases is displayed within the bar. P>0.05 (ns); P<0.05 (*); P<0.01 (**); P<0.001 (***). **(B)** Top: experimental design. An acute pancreatitis was induced in 40 week-old *Kras** and *Kras*; Gata4*$^{P-/-}$ mice; analysis was performed at 1 year of age. Bottom: same histopathological analysis as in panel A (n=7 for *Kras*;* n=6 for *Kras*;Gata4*$^{P-/-}$).

However, in none of these settings did we observe an increased incidence of PanIN in Gata4-null context, with only 2/8 "early" and 1/6 "late" mice presenting 1 focus of PanIN. Surprisingly, PDAC incidence in *Kras**;*Gata4*$^{P-/-}$ mice was lower than in control mice: in the "early" pancreatitis experiment we did not observe any tumour (0/8), and in "late" pancreatitis we observed reduced frequency (33.33% cases; 2/6) compared to *Kras** mice of the same experiment (85.71% cases; 6/7), but a similar frequency compared to non-challenged 1-yer-old mice (35.71% cases; 5/14) (Fig. 7B, Fig. 8A, 8B).

For the rest of the analyses, the results followed the same trend as in Fig. 7: we did not observe AFL in *Kras*;Gata4*$^{P-/-}$ mice; and lipomatosis was increased in the absence of Gata4, as described earlier. Of note, the low number of mice used in these experiments (4 *Kras**; 8 *Kras**;*Gata4*$^{P-/-}$ mice in "early" settings; 7 *Kras**; 6 *Kras*;Gata4*$^{P-/-}$ mice in "late" settings) hampers a definitive interpretation of the results, and the only solid conclusion we draw is that an episode of acute pancreatitis does not increase PanIN formation in *Kras*;Gata4*$^{P-/-}$ mice.

2.3. PDAC initiation from adult acinar cells results in a similar phenotype upon Gata4 inactivation

Previous studies have shown that PanINs and PDAC can arise in mice in which *Kras** activation takes place in adult acinar cells provided that there is pancreatitis-induced damage. To determine whether PDAC can be initiated from acinar cells in *Kras*;Gata4*$^{-/-}$ mice , we induced *Kras** activation and *Gata4* deletion in acinar cells of LSL-*Kras*G12V$^{+/KI}$; *Gata4*$^{lox/lox}$; *Ptf1a*-CreERT2$^{+/KI}$; *Rosa26*-YFP$^{KI/KI}$ mice at 8 weeks of age, when Ptf1a is only expressed in acinar cells (termed i-*Kras*;Gata4*$^{-/-}$ and i-*Kras** as controls, where "i" stands for inducible).

In order to promote carcinogenesis, we induced chronic damage by administering one supramaximal dose of caerulein daily for 6 months and further allowing mice to age until 1 year, when we analysed the histology (Fig. 9A). Tumours from both i-*Kras** and i-*Kras*;Gata4*$^{P-/-}$ mice showed ductal morphology and YFP positive staining, demonstrating that acinar cells are indeed the cell of origin of the PDACs (Fig. 9B). These tumours appeared to be proliferative as shown by enhanced Ki67 staining within the tumoural cells. Again, we observed multiple PanIN foci in i-*Kras** mice but not in i-*Kras*;Gata4*$^{-/-}$ pancreata that appeared to be YFP+, also indicating an acinar origin, which presented a low rate of Ki67+ cells (Fig. 9B). Importantly, we found that PDAC incidence in both strains was not significantly different (P=0.622) (Fig. 9C).

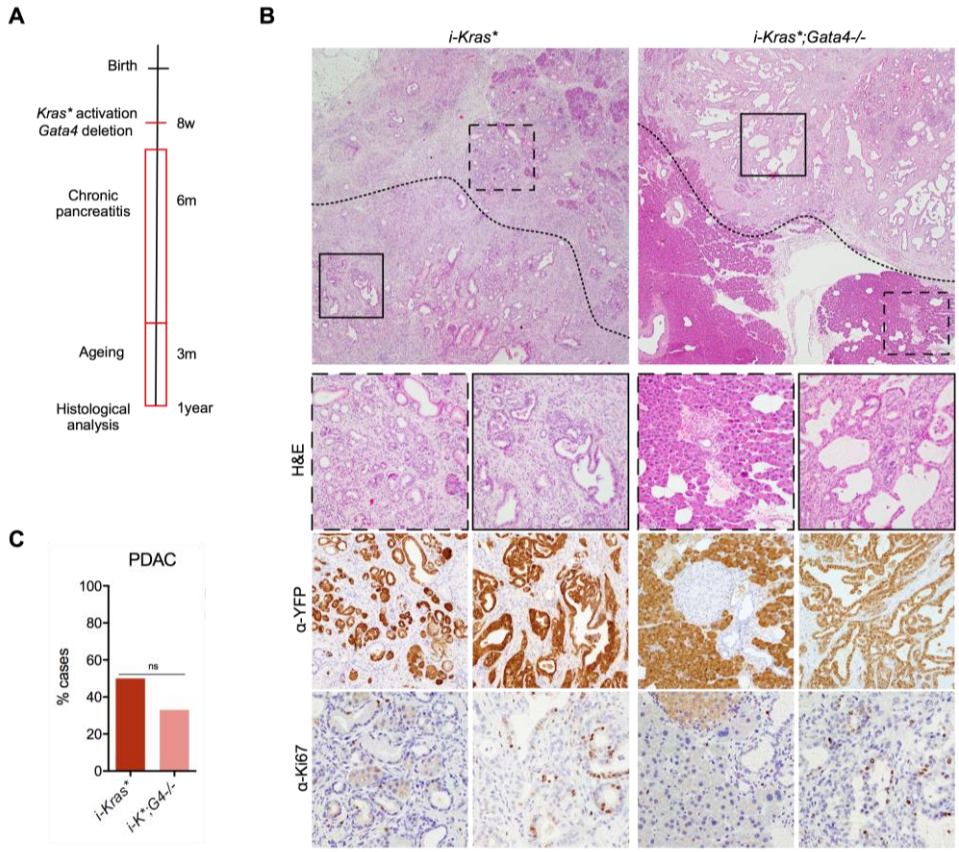

Figure 9. Acinar cells give rise to PDAC in i-*Kras and i-*Kras**;*Gata4*$^{P-/-}$ mice. (A)** Experimental design: two weeks after inducing recombination, a chronic pancreatitis was induced by injecting one supramaximal dose of caerulein daily for 6 months. Three months later mice were sacrificed for histological analysis. **(B)** Low-magnification representative images of i-*Kras** (left) and i-*Kras*;Gata4*$^{P-/-}$ mice (right) pancreata. Dotted square includes non-tumoural areas, while normal square includes a region with PDAC, which are magnified below. Detection of YFP by IHC in PanINs and the epithelial compartment of the tumour indicates an acinar origin. Ki67 staining shows low proliferation in non-tumoural areas and high proliferation in cancer. **(C)** Quantification of tumour incidence in i-*Kras** and i-*Kras**;*Gata4*$^{P-/-}$ mice showing no significant differences between the two groups (P=0.622).

To specifically study PanIN formation upon loss of Gata4 in adult acinar cells, we induced recombination in i-*Kras**;*Gata4*$^{P-/-}$ mice and controls at 8 weeks of age and administered caerulein over 2 months to induce a chronic pancreatitis. Mice where sacrificed at 20w of age and the pancreas was analysed (Fig. 10A). We observed an extensive presence of PanIN in i-*Kras** mice, affecting entire lobules in some cases (Fig. 10B). In contrast, we did not observe any PanIN in i-*Kras**;*Gata4*$^{P-/-}$ mice (Fig. 10B). Regarding remodelling, we observed areas of ADM similar to the ones observed in *Gata4*$^{P-/-}$ mice upon acute pancreatitis (Fig. 6B). We used image analysis to quantify the proportion of the pancreatic parenchyma undergoing remodelling/metaplasia and found involvement of

55.6% ±26.11 vs. 13.9% ±4.9 in i-*Kras**; *Gata4*$^{P-/-}$ of the area in i-*Kras** vs. i-*Kras**; *Gata4*$^{P-/-}$ mice, respectively (P=0.009) (Fig. 10C). Altogether, these results indicate that Gata4 is required for adult acinar cells to form PanINs but not to develop PDAC.

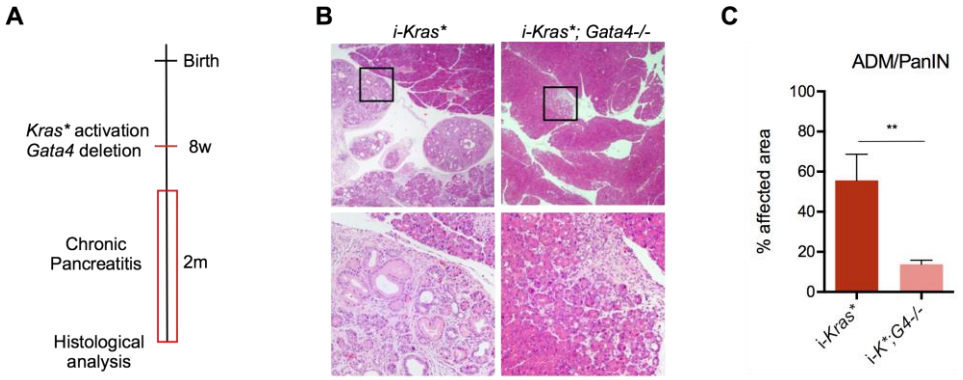

Figure 10. i-*Kras;*Gata4*$^{-/-}$ adult acinar cells are refractory to ADM/PanIN induction. (A)** Schematic representation of experimental design. *Kras** activation and *Gata4* deletion were achieved in 8-week old mice by TMX administration using the *Ptf1a*-CreERT2 allele, resulting in acinar-specific recombination. Recombined i-*Kras** and i-*Kras**;*Gata4*$^{-/-}$ mice underwent a chronic caerulein pancreatitis for two months and sacrificed for histological analysis. **(B)** Representative H&E staining showing a higher percentage of affected area in i-*Kras** mice compared to i-*Kras**;*Gata4*$^{-/-}$ upon induction of chronic pancreatitis. Affected area includes ADM and PanIN lesions. **(C)** Quantification of affected area in each genotype (P=0.009).

2.4. IL17 rescues *Gata4*$^{-/-}$ defect to undergo ADM *in vitro*, but it induces abnormal metaplasia and lipomatosis instead of PanINs *in vivo.*

The *in vivo* experiments described above clearly show that Gata4 is necessary for pancreatic cells to undergo *Kras**-driven metaplasia and form PanINs.

ADM can be largely recapitulated *in vitro* using primary acinar cell preparations cultured in 3D. To determine whether Gata4 is also required for *in vitro* ADM, we isolated acinar cells from *Gata4*$^{-/-}$ and control pancreata and embedded them in matrigel. As has been previously described (Means *et al.*, 2005), control cells spontaneously underwent ADM and formed cystic structures that could be visualized under the microscope, albeit at low frequency. The ability of *Gata4*$^{-/-}$ acinar cells to form cysts was dramatically reduced (P<0.001) (Fig. 11A).

EGF and several other cytokines such as IL17, IL6, IL1b have been shown to promote ADM (Ardito *et al.*, 2012; Ma *et al.*, 2009; Mcallister *et al.*, 2014; Zhang *et al.*, 2013); therefore, we analysed their effect on control and *Gata4*$^{-/-}$ acinar cells *in vitro*. We prioritized cytokines that are induced upon activation of *Kras** in the pancreas (upregulated in an RNAseq data comparing *Kras** vs control pancreas) since they are candidates to play a role in neoplastic transformation

(Fig. 11A). As expected, EGF significantly increased 3.5-fold the number of cysts with respect to the non-treated controls (P<0.0001). EGF also induced cyst formation in *Gata4*$^{-/-}$ acinar cells compared with non-treated *Gata4*$^{-/-}$ acini (6.25-fold, P=0.053), yet the number of cysts remained significantly lower than in EGF-treated controls (P<0.0001). As expected, IL17 significantly increased the number of cysts (P<0.0001) and, in addition, it rescued almost completely the defect observed in *Gata4*$^{-/-}$ acinar cells to levels similar to control (P=0.307). IL6 slightly increased the number of cysts in control acinar cell preparations (P=0.033) but it had no effect on the number of cysts formed by *Gata4*$^{-/-}$ cells. The remaining cytokines tested, including IL1b, IL2, IL4, IL10, Ccl5, Cxcl12, and Reg3b, did not have any significant effect on cyst formation in control or *Gata4*$^{-/-}$ cells (Fig. 11A). Altogether, these results support the *in vivo* observations that Gata4 plays a crucial role in regulating ADM and show that IL17 can rescue this defect. Furthermore, these findings establish that Gata4-null cells can undergo ADM under certain circumstances.

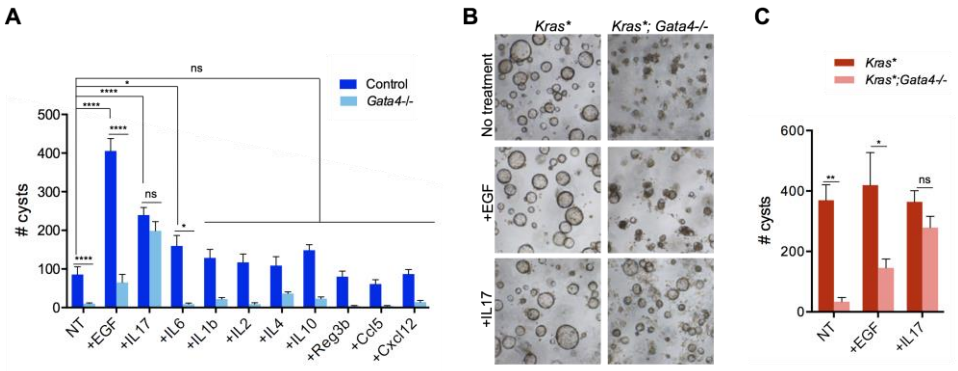

Figure 11. *Gata4*$^{-/-}$ acinar cells do not undergo ADM *in vitro* spontaneously and this defect is rescued upon IL17 treatment (A) Acinar cells from control and *Gata4*$^{P-/-}$ mice were embedded in matrigel and the ability of a panel of cytokines to rescue the lack of ADM in *Gata4*$^{-/-}$ cells was tested. After 5 days of culture, only EGF, IL17 and IL6 significantly increased the number of cysts compared to non-treated acinar cells. Specially, EGF and IL17 induced the formation of big, rounded cystic structures. Experiments/condition: n=13 for NT, EGF, IL17; n=7 for IL6, IL1b; n=3-5 for IL2, IL4, IL10, Reg3b, Ccl5, Cxcl12. P>0.05 (ns); P<0.05 (*); P<0.01 (**); P<0.001 (***); P<0.0001 (****). **(B)** *Kras** and *Kras**;*Gata4*$^{-/-}$ acinar cells were embedded in matrigel and treated with EGF and IL17. Left panel, representative images of the cells after 5 days of culture. Right panel, quantification of number of cysts, showing that in basal conditions, and when treated with EGF, *Gata4*$^{-/-}$ cells ability to undergo ADM is dramatically reduced (P<0.001). In contrast, IL17 efficiently rescues cyst formation although their size is smaller. N=5 experiments for each condition.

The lack of PanINs described above was identified in a *Kras** background. Therefore, we also conducted the *in vitro* ADM assay with *Kras** acinar cells. *Kras** acini spontaneously formed a nearly 4-fold higher number of cysts compared to *Kras*^{WT} cells (Fig. 11A, 11B). Similar to Kras^{WT};*Gata4*$^{-/-}$ counterparts, *Kras**;*Gata4*$^{-/-}$ cells showed very reduced ability to form cysts in basal conditions (P=0.008 compared to non-treated *Kras** acini). Upon EGF treatment, a modest

increase was observed both in control and *Kras*^{WT};*Gata4*$^{-/-}$ cells (P=0.031 compared to EGF-treated *Kras** acini). Again, IL17 was able to increase the number of cysts formed by *Kras**;*Gata4*$^{-/-}$ cells to the levels of control cells (P=0.309), as observed in *Kras*^{WT} background (Fig. 11B).

Interestingly, IL17 has been described to induce PanIN formation when expressed from adenoviruses coding for IL17 injected orthotopically in *Kras** pancreas (Mcallister *et al*., 2014). To determine whether adenoviral delivery of IL17 *in vivo* would be able to induce PanINs in *Kras**;*Gata4*$^{P-/-}$ mice, we injected adenovirus preparations expressing either IL17 or control GFP into the pancreas of 10-week-old control *Kras** and *Kras**;*Gata4*$^{P-/-}$ mice and performed histopathological analysis 6 weeks after inoculation. Mice injected with adeno-GFP showed a slight increase in lipomatosis in *Kras**;*Gata4*$^{-/-}$, in accordance with previous analyses (Fig. 12A). As described in McAllister, *et al.,* we observed that *Kras** mice injected with adeno-IL17 presented classical PanIN lesions expressing intestinal and gastric mucins (Fig. 12B).

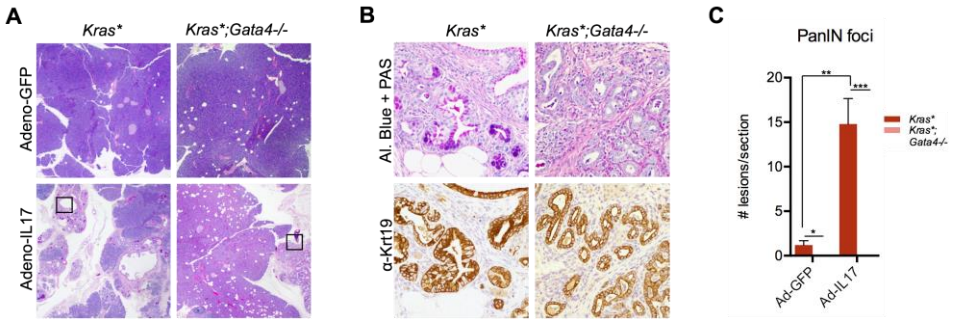

Figure 12. IL17 induces abnormal metaplasia and lipomatosis instead of PanINs in *Kras*;Gata4*$^{-/-}$ pancreas. *Kras** and *Kras**;*Gata4*$^{P-/-}$ mice were orthotopically injected with 10^9 PFU adenoviruses expressing either Il17 or GFP (n=5 for each condition). Mice were sacrificed 6 weeks after surgery and the histology was analysed for the presence of low-grade PanIN using Alcian Blue/PAS staining to reveal the presence of mucins. **(A)** Upper images show pancreata injected with control adeno-GFP. Lower images show extended areas with PanIN lesions in *Kras** (left), and lack of PanIN in *Kras**;*Gata4*$^{P-/-}$, with an increase of lipomatosis instead (right). **(B)** High-magnification images showing PanIN lesions stained for mucins in *Kras**, and the presence of ductal-like structures -which are not PanIN due to lack of mucin production- in *Kras**;*Gata4*$^{P-/-}$ pancreas. In both cases the presence of Krt19+ staining indicate a ductal nature of the cells. **(C)** Quantification of PanINs assessed by presence of mucins within the lesions, showing a significant increase in *Kras** mice injected with Ad-IL17 compared to Ad-GFP (P=0.0015). *Kras**;*Gata4*$^{P-/-}$ mice did not present any mucinous lesion both when injected with Ad-GFP (P=0.04 compared to *Kras**) and Ad-IL17 (P<0.001 compared to *Kras**).

Quantification of PanIN foci determined a significant increase in Ad-IL17 treated *Kras** mice compared to Ad-GFP (P=0.0015) (Fig. 12C). In contrast, *Kras**;*Gata4*$^{-/-}$ pancreata completely lacked PanINs in both conditions (compared to *Kras** mice, P=0.04 for Ad-GFP, and P<0.001 for Ad-IL17 inoculation) (Fig. 12C). Instead they

presented with areas of abnormal non-mucinous metaplasia and extensive lipomatosis (Fig. 12A,B). The atypical lesions identified in *Gata4*$^{-/-}$ pancreata expressed Krt19 (Fig. 12B), suggesting that IL17 induces formation of ductal-like structures *in vivo* as well as *in vitro.* However, the lack of mucins indicates that these are not classical PanINs (Fig.12B) and formally demonstrates that Gata4 is necessary to establish and maintain the PanIN phenotype.

2.5. *Kras*;Gata4*$^{-/-}$ pancreata harbour unconventional metaplastic lesions lacking phospho-Stat3 signalling

The lack of PanIN lesions in mice developing PDAC suggests that an alternative type of preneoplastic lesion develops in *Kras*;Gata4*$^{-/-}$ mice that may not have been recognized previously. Pathological analysis of the pancreas of *Kras*;Gata4*$^{-/-}$ mice at 1 year of age revealed focal regions of acinar cell clusters characterized by paleness, reduced eosinophilia, and a certain degree of hyperplasia with or without enlarged lumina, suggesting altered differentiation (Fig. 13). We designate these lesions "PALes" for "Pale Acinar Lesions", and we hypothesize that PALes are clusters of acinar cells initiating *Kras**-induced metaplastic process, which normally progress into ADM/PanIN in a context of Gata4$^{+/+}$, but in a context of Gata4$^{-/-}$ form unconventional metaplastic lesions -or undergo adipocyte transdifferentiation. A thorough pathological analysis of *Kras** pancreata also disclosed the occurrence of PALes, as well as well-recognized ductal lesions (ADM and PanIN) (Fig. 13). Indeed, other reports have previously noticed the presence of hyperplastic clusters of acini in Kras* pancreata (Guerra *et al*., 2007, supplemental data). To identify and characterize PALes at the molecular level, we used immunohistochemistry with a panel of antibodies. Results are summarized in Table 2.

	Acini		**PALe**		**Ductal-like**	
	*Kras**	*K*;G4*$^{-/-}$	*Kras**	*K*;G4*$^{-/-}$	*Kras** (PanIN)	*K*;G4*$^{-/-}$ (D-PALe)
Krt19	-	+	++	-	+++	+++
Sox9	-	-	+	++	+++	+++
Hnf1b	-	-	-	-	+++	+
p65	-	-	+++	++	+	+
phospho-STAT3	-	-	+++	-	+	-

Table 2. Comparative analysis of epithelial and signalling markers assessed in lesions from 1-year-old *Kras** and *Kras*;Gata4*$^{-/-}$ (*K*;G4*$^{-/-}$) mice. D-PALe: ductal PALe.

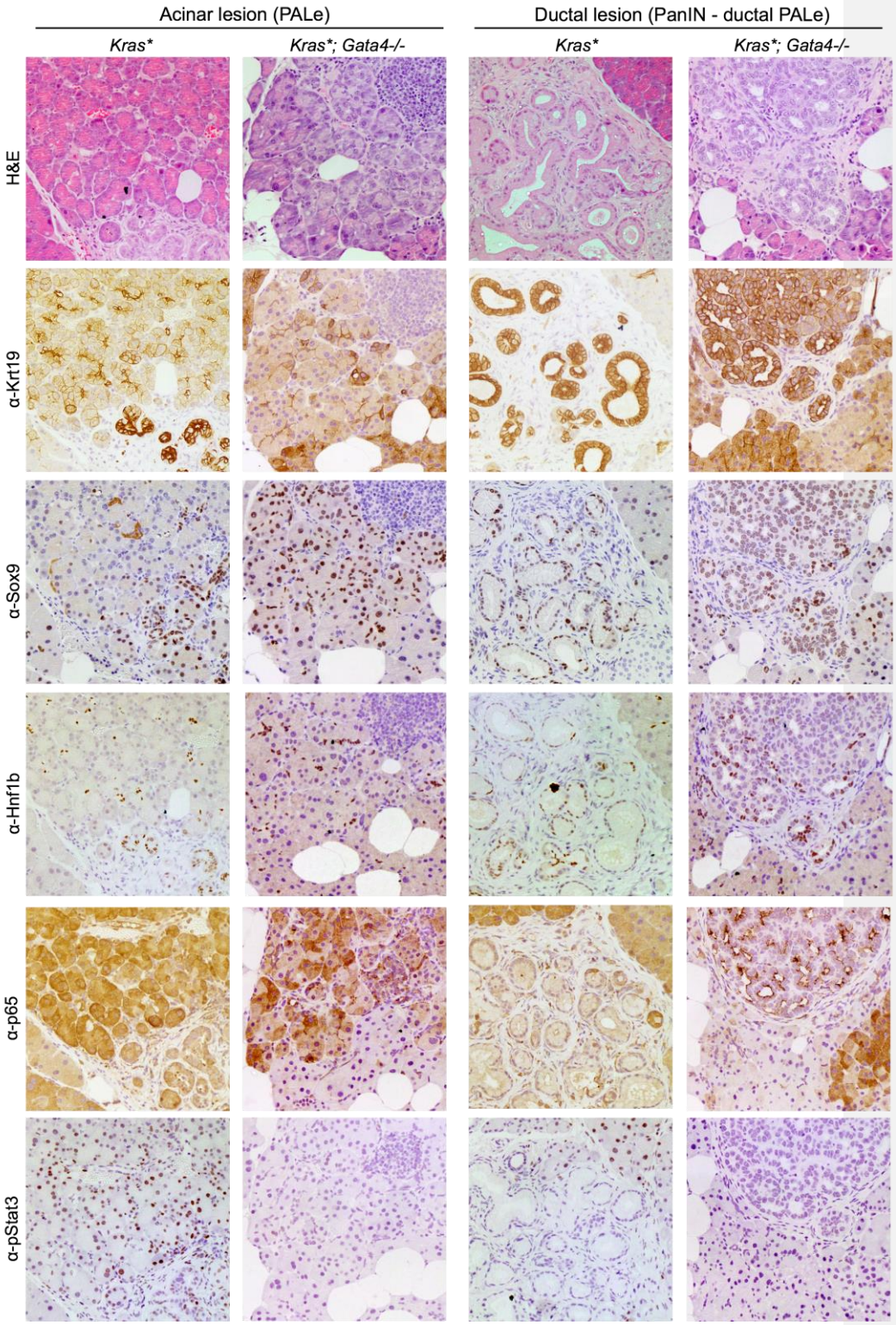
Acinar lesion (PALe)
Ductal lesion (PanIN - ductal PALe)
Kras*
Kras*; Gata4-/-
Kras*
Kras*; Gata4-/-
H&E
α-Krt19
α-Sox9
α-Hnf1b
α-p65
α-pStat3

Figure 13. Kras*;*Gata4*$^{P-/-}$ mice develop unconventional metaplastic lesions that display differential activation of signalling pathways. Representative images of serial sections of pancreata from *Kras** and Kras*;*Gata4*$^{P-/-}$ mice, showing acinar and ductal features. ADM was assessed using Krt19, Sox9 and Hnf1b. Signalling pathways important during transformation, including NF-kB and Jak-Stat-, were assessed using phospho-Stat3 (pStat3) and p65, respectively.

ADM markers

We first analysed expression of Sox9 and Krt19, proteins that are expressed "early" in the metaplastic process. In addition, we analysed Hnf1b, which is expressed in established ductal structures. In *Kras** mice, PALes showed slightly increased expression of Krt19 compared to normal acinar cells. In lesions where complete ductal reprogramming has occurred (ADM, PanIN) expression of Krt19 was further enhanced. In contrast, PALes in *Kras*;Gata4*$^{-/-}$ did not display marked Krt19 up-regulation, and only when Gata4-null lesions had acquired a ductal morphology did they show Krt19 levels similar to those of in PanINs (Fig. 13). In *Kras** mice, Sox9 was expressed in PALes as well as in all ductal lesions. Similarly, Sox9 was expressed in PALes in *Kras*;Gata4*$^{-/-}$ as well as in ductal lesions (Fig. 13), suggesting that Sox9 functions upstream of -and its expression is not affected by- Gata4 loss. As expected, in *Kras** mice, Hnf1b was not expressed in *Kras** PALes and it was only detected in PanINs. Similarly, Hnf1b was not detected in *Kras*;Gata4*$^{-/-}$ PALes but it was heterogeneously present in ductal lesions, even within a given duct (Fig. 13).

Signalling molecules

In order to shed light on the status of the Nf-kb and Jak/Stat3 signalling pathways in each type of lesion, we stained the pancreata of 1-year old mice for p65 and phospho-Stat3 (Fig. 13). In *Kras** mice, PALes showed high p65 staining, unlike PanINs. Similarly, we observed up-regulation of p65 in Kras*;*Gata4*$^{-/-}$ PALes, but not in ductal lesions.

Phospho-Stat3 staining followed a similar pattern since PALes in *Kras** mice expressed high levels of phospho-Stat3, and also some PanINs, although at lower levels than PALes. In contrast, phospho-Stat3 was undetectable in PALes of Kras*;*Gata4*$^{-/-}$ mice, this being the most striking difference between genotypes for all markers tested. Similarly, phospho-Stat3 was undetectable in the ductal lesions present in Gata4-null pancreata. These results suggest that Gata4 is necessary for proper *Kras**-induced signalling during metaplastic transformation, especially through Stat3.

2.6. Gata4 participates in Kras*-induced inflammation

In order to get insight into the mechanisms regulated by Gata4 in a *Kras** context prior to acinar transformation and PanIN formation, we performed RNAseq to

analyse the transcriptome of 8 week-old *Kras** (n=3) and *Kras*;Gata4*$^{-/-}$ (n=3) pancreata, when metaplastic processes have not been widely initiated, and very few lesions can be detected. The RNAseq data obtained from *Ptf1a*-Cre$^{+/KI}$ and *Gata4*$^{P/-}$ mice were used as controls (Fig. 14).

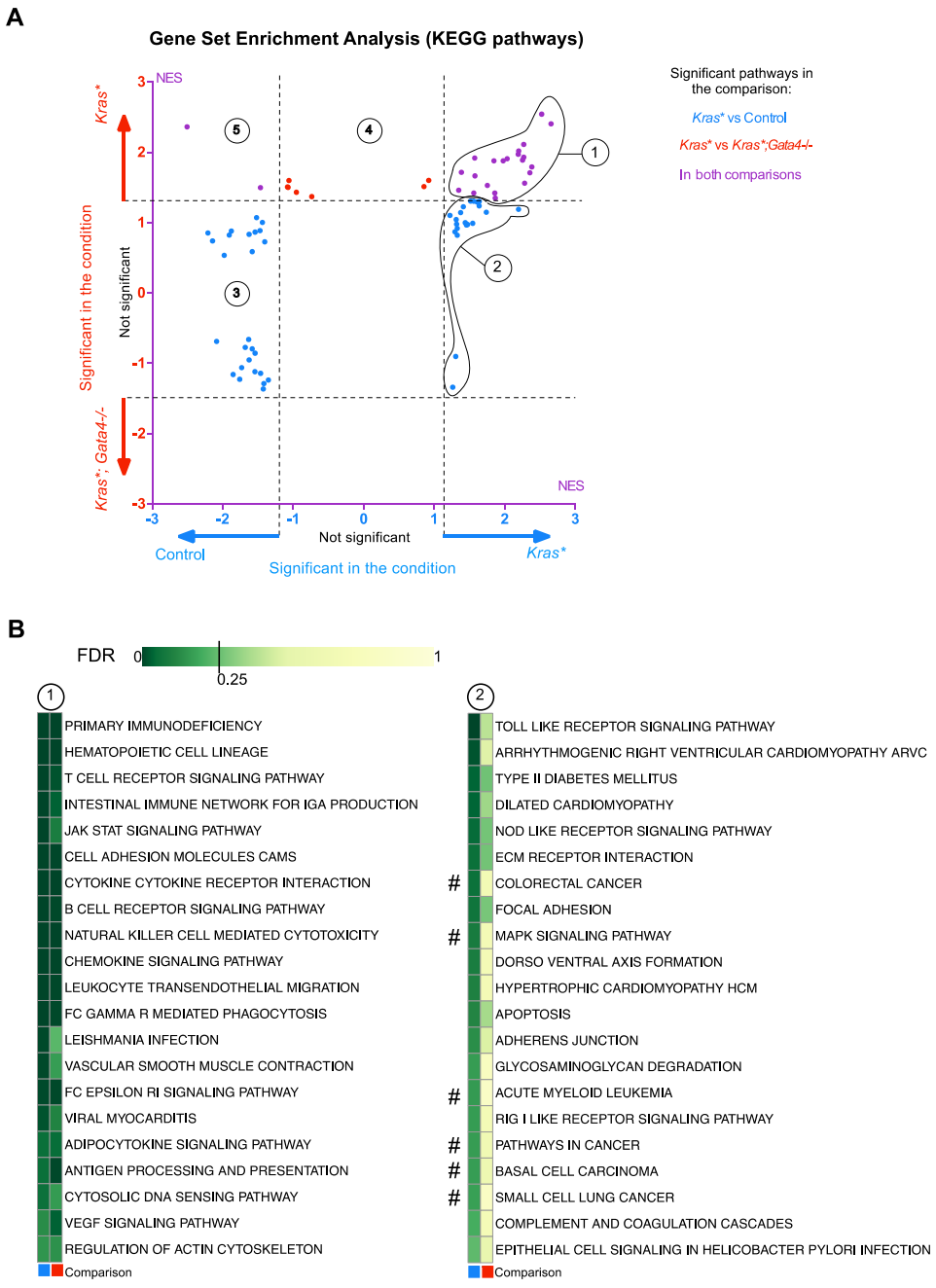

Figure 14. Kras*-induced inflammation requires functional Gata4. (A) RNA-seq data from pancreatic tissue of 8 week-old control, *Kras** and *Kras*;Gata4*$^{-/-}$ (*K*;G4*$^{-/-}$*)* mice was analysed to determine the consequences of *Gata4* loss in a *Kras** background. Gene Set Enrichment Analysis

(Fig. 14 cont.) (GSEA) was performed on the comparisons "Control vs *Kras**" and " *Kras**;*Gata4*$^{-/-}$ vs *Kras**". We applied the KEGG library, which includes a variety of signatures that are representative for the majority of cellular processes in physiological and pathological conditions. Pathways that appeared to be differentially activated with FDR<0.25 were plotted. The values of the axis correspond to the Normalized Enrichment Score (NES). Different subgroups of pathways are indicated for further description. **(B)** Heat map showing FDR values of the two groups of pathways underscored in Fig. 14A. Left panel shows the pathways that are significantly enriched in *Kras** when compared with both control and *Kras**;*Gata4*$^{-/-}$, which are mostly related to inflammation. Right panel shows the pathways that are significantly enriched in the comparison "*Kras** vs Control", being these upregulated in Kras* condition, which include several pathways related to cancer (#). Name of pathways in subgroups 3, 4 and 5 are displayed in Table 3.

Our first approach consisted of performing GSEA on the following comparisons: (I) control (*Ptf1a*-Cre$^{+/KI}$) vs *Kras** transcriptome, to unravel the biological processes and pathways induced upon Kras activation; (II) *Kras** vs *Kras*; Gata4*$^{-/-}$ transcriptome, to determine the pathways downregulated in the absence of Gata4 that might contribute to the reduced ADM and absence of PanINs. We applied the KEGG library and found that a similar set of pathways was significantly enriched in *Kras** when compared to both control and *Kras*;Gata4*$^{-/-}$ pancreas (Fig. 14A). Several of these pathways were related to immune system activation and inflammatory processes (Fig. 14B), indicating that Kras* induces an early inflammatory response that is absent upon Gata4 deletion and that is not required for PDAC development.

Kras* signals through the activation of MAPK and a constitutive activation of this pathway is linked to tumour formation in many tissues. In accordance, "MAPK signalling pathway" as well as other pathways related to carcinogenesis such as "Colorectal cancer", "Acute myeloid leukemia", "Pathways in cancer", "Basal cell carcinoma" and "Small cell lung cancer" were upregulated in *Kras** compared to controls. Importantly, these pathways were also upregulated in *Kras*;Gata4*$^{-/-}$ pancreata, indicating that they are likely relevant to PDAC formation but they do not inevitably lead to PanINs (Fig. 14B). The list of the pathways enriched in other comparisons is displayed in Table 3.

These results suggest that Gata4 controls, directly or indirectly, the Kras*-driven inflammatory response. In order to characterize this phenotype in more detail, we considered the expression of genes related to inflammation as well as cell-specific markers using the RNAseq data (Fig. 15A). We observed that several chemoattractants (*Ccl19, Ccl21a, Ccl5*) and adhesion molecules (*Icam1, Cd2, Itgal, Itgb7, Sell*) were significantly upregulated in *Kras** compared to control pancreata and also compared to *Kras*;Gata4*$^{-/-}$ pancreas. Accordingly, we observed a significant increase in total Cd45+ leukocytes. Analysis of specific leukocyte subpopulations revealed no significant differences in the neutrophil marker *Mpo*. Contrary, we observed a significant upregulation of *Cd3e*, *Cd4*, *Cd8a*, *Foxp3* (T lymphocytes) and *Cd19*, *Pax5*, *Cd22* (B lymphocytes) in *Kras** pancreas compared to the other conditions. Interestingly, the expression of macrophage

markers (F4/80, Mac2, *Csfr1*, *Clec10a*) did not differ between control and *Kras** pancreata, but levels significantly decreased in *Kras**;*Gata4*$^{-/-}$ samples (Fig. 15A). These results suggest that Gata4 modulates the presence of resident macrophages in the pancreas, as this is the only immune subpopulation that significantly decreases in Gata4-null pancreata, independently of *Kras* status.

[3]

Alanine aspartate and glutamate metabolism	Long term potentiation
Alzheimer's disease	Oxidative phosphorylation
Aminoacyl tRNA biosynthesis	Parknison's disease
Amyotrophic lateral sclerosis (ALS)	Peroxisome
Arginine and proline metabolism	Propanoate metabolism
Butanoate metabolism	Proteasome
Cardiac muscle contraction	Protein export
Cysteine and methionine metabolism	Regulation of autophagy
Fatty acid metabolism	RNA polymerase
Glutathione metabolism	Selenoamino acid metabolism
Glycolysis gluconeogenesis	Spliceosome
Histidine metabolism	Valine leucine and isoleucine degradation
Huntington's disease	Vasopressin regulated water reabsorption

[4]

Arachidonic acid metabolism
Ether lipid metabolism
Galactose metabolism
Glycerolipid metabolism
Lysosome
PPAR signaling pathway
Prion diseases

[5]

Ribosome
Systemic lupus erythematosus

Table 3. KEGG pathways in Figure 14A. [3] Pathways significant only in the comparison *Kras** vs Control, upregulated in Control. [4] Pathways significant only in the comparison *Kras** vs *Kras*;Gata4*$^{-/-}$, upregulated in *Kras*.* [5] Pathways significant in both comparisons, enriched in *Kras** (vs *Kras*Gata4*$^{-/-}$) and in Control (vs *Kras**).

The RNAseq data showed that the Kras*-induced inflammatory response is blunted in the absence of Gata4. In order to unravel which pro-inflammatory factors are expressed by *Kras** acinar cells in a Gata4-dependent manner, we analysed isolated acini (Fig. 15B). First, we examined the presence of contaminating T lymphocytes as a quality control for acinar explant purity by assessing expression of CD3 and found very low levels of expression with no significant differences between the two conditions. Then, we assessed the expression of genes that may induce an inflammatory response, whose expression was significantly downregulated in the RNAseq data in *Kras**;*Gata4*$^{-/-}$

compared to *Kras** pancreata, including *Ccl5, Ccl19, Icam1, Cxcl12, Cxcr4* and *Reg3b*.

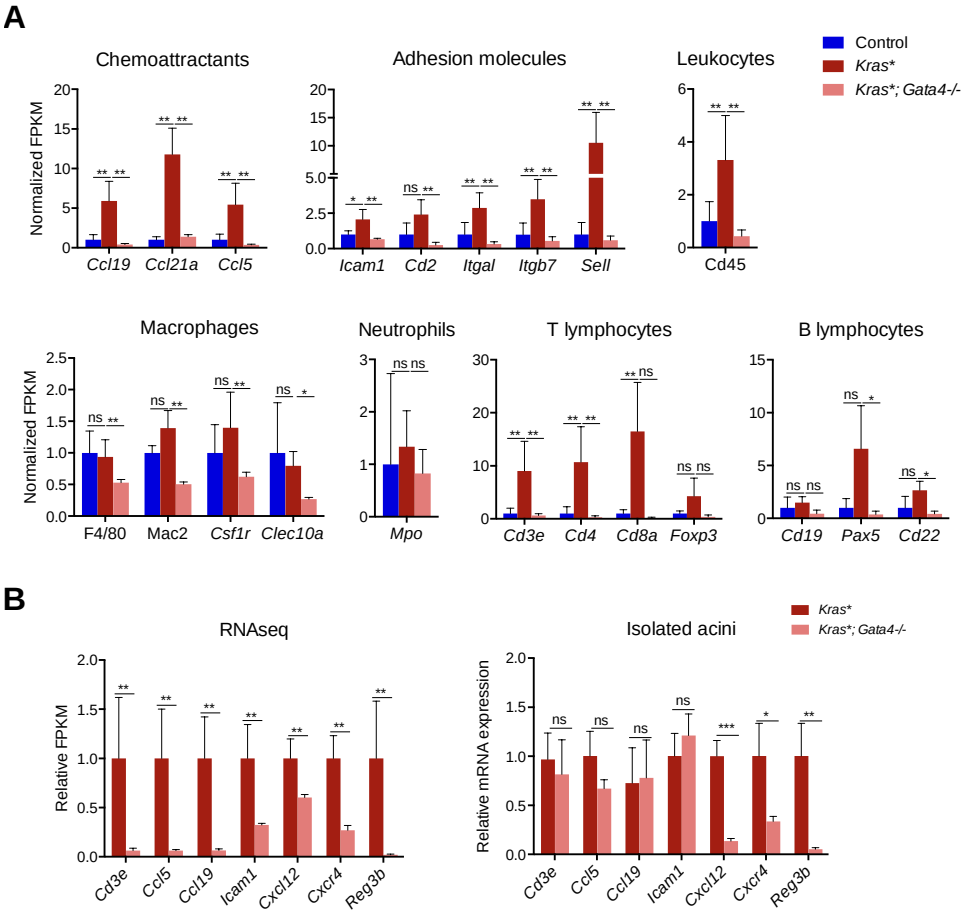

Figure 15. Gata4 regulates the acinar-specific expression of some inflammation-related factors (A) RNA-seq results showing expression of genes coding for chemoattractants, adhesion molecules, and markers for all leukocytes (Cd45, coded by *Ptprc*), and specific leukocyte types (macrophages, neutrophils, T and B lymphocytes). In general, expression of all markers is higher in *Kras** than in both control and *Kras**;*Gata4*$^{P-/-}$ mice. P>0.05 (ns); P<0.05 (*); P<0.01 (**); P<0.001 (***). For the following markers, the gene coding for the specified protein is: *Adgre1* (F4/80); *Lgals3* (Mac2). **(B)** Lef panel: Gene expression of inflammation-related genes in freshly isolated acini cultured for 24h culture, including T lymphocyte markers (*Cd3e*) for negative control, chemoattractants (*Ccl5, Ccl19*), adhesion molecules (*Icam1*), the pro-inflammatory *Cxcl12-Cxcr4* axis, and the gene coding for pancreatitis-associated protein (*Reg3b*). All tested genes are expressed at significantly lower levels in *Kras**;*Gata4*$^{P-/-}$ than in *Kras** pancreas based on the results of the RNA-seq analysis (left panel). However, only *Cxcl12*, *Cxcr4*, and *Reg3b* are significantly decreased in *Kras**;*Gata4*$^{P-/-}$ isolated acini (right panel).

In isolated *Kras**;*Gata4*$^{-/-}$ acini, expression of *Ccl5*, *Ccl19* and *Icam1* was not different from *Kras** acini, indicating that differences in the levels of these chemoattractants in the RNAseq data is not due to an epithelial cell-autonomous

defect, but likely due to improper activation of immune subpopulations, instead. In contrast, *Cxcl12*, *Cxcr4* and *Reg3b* (also termed Pancreatitis Associated Protein) transcripts were significantly downregulated in *Kras**;*Gata4*$^{-/-}$ isolated acini, suggesting that Gata4 controls their expression, directly or indirectly, at the epithelial cell level (Fig. 15B). Importantly, these genes have been described to be expressed during inflammatory processes in the pancreas. These results show that changes in gene expression in the RNAseq data are not only due to deregulated transcription in Gata4-null acinar cells, but also in other cell types -most likely leukocyte subpopulations-, whose gene expression is also affected by the altered signalling coming from *Gata4*$^{-/-}$ acini.

2.7. Infiltration of Kras*-induced epithelial lesions by macrophages and T lymphocytes requires Gata4

In order to validate the RNAseq results showing a decrease in inflammatory cells in 8-week-old *Kras**;*Gata4*$^{-/-}$ pancreata, we stained for F4/80, Mpo, Cd3, and Pax5 to assess the presence of macrophages, neutrophils, T lymphocytes and B lymphocytes, respectively. We did not observe differences in overall infiltrating cells in acinar parenchyma, but we noticed a differential presence of leukocytes surrounding histological abnormalities (lesions). We therefore analysed early lesions, including PALes and ductal metaplasias, from 4 different *Kras** (14 lesions) and 4 *Kras*;Gata4*$^{-/-}$ (16 lesions) pancreata (Fig. 16).

We observed that macrophages are present in all Kras*-induced lesions (14/14), both PALes and ductal lesions. Accordingly, other reports have described macrophages to be the most abundant leukocyte subpopulation in Kras*-induced lesions (Liou & Storz, 2015). In contrast, lower presence of macrophages could be observed in PALes and ductal lesions in *Kras*;Gata4*$^{-/-}$ pancreas (P=0.028). T lymphocytes were present in nearly all PALes and ductal lesions in *Kras** (12/14), but we observed fewer *Kras**;*Gata4*$^{P-/-}$ lesions with T lymphocyte infiltration (10/16), which also presented lower T cell abundance (P<0.001). Neutrophils could only be observed in nearly half (6/14) of the ductal lesions that were analysed and in none of the PALes; by contrast, only one *Kras*;Gata4*$^{-/-}$ lesion subjected to analysis showed presence of neutrophils (P=0.229). Finally, B lymphocytes were absent in *Kras** mice, and only one *Kras**;*Gata4*$^{-/-}$ lesion presented positive staining (P=0.341) (Fig. 16A). Altogether, these data suggest that Gata4 plays a role in recruiting macrophages and T lymphocytes to lesions in *Kras** pancreata, and its lack results in decrease infiltration of these leukocytes.

We also examined the presence of macrophages, T lymphocytes, B lymphocytes and neutrophils in the mesenteric lymph nodes that surround the pancreas and found that macrophages were nearly absent in *Kras**;*Gata4*$^{P-/-}$ compared to *Kras** mice (P<0.0001) (Fig. 16B). For the other leukocyte subpopulations we did not

observe major differences. Altogether, these data suggest that lack of Gata4 impairs macrophage mobilization.

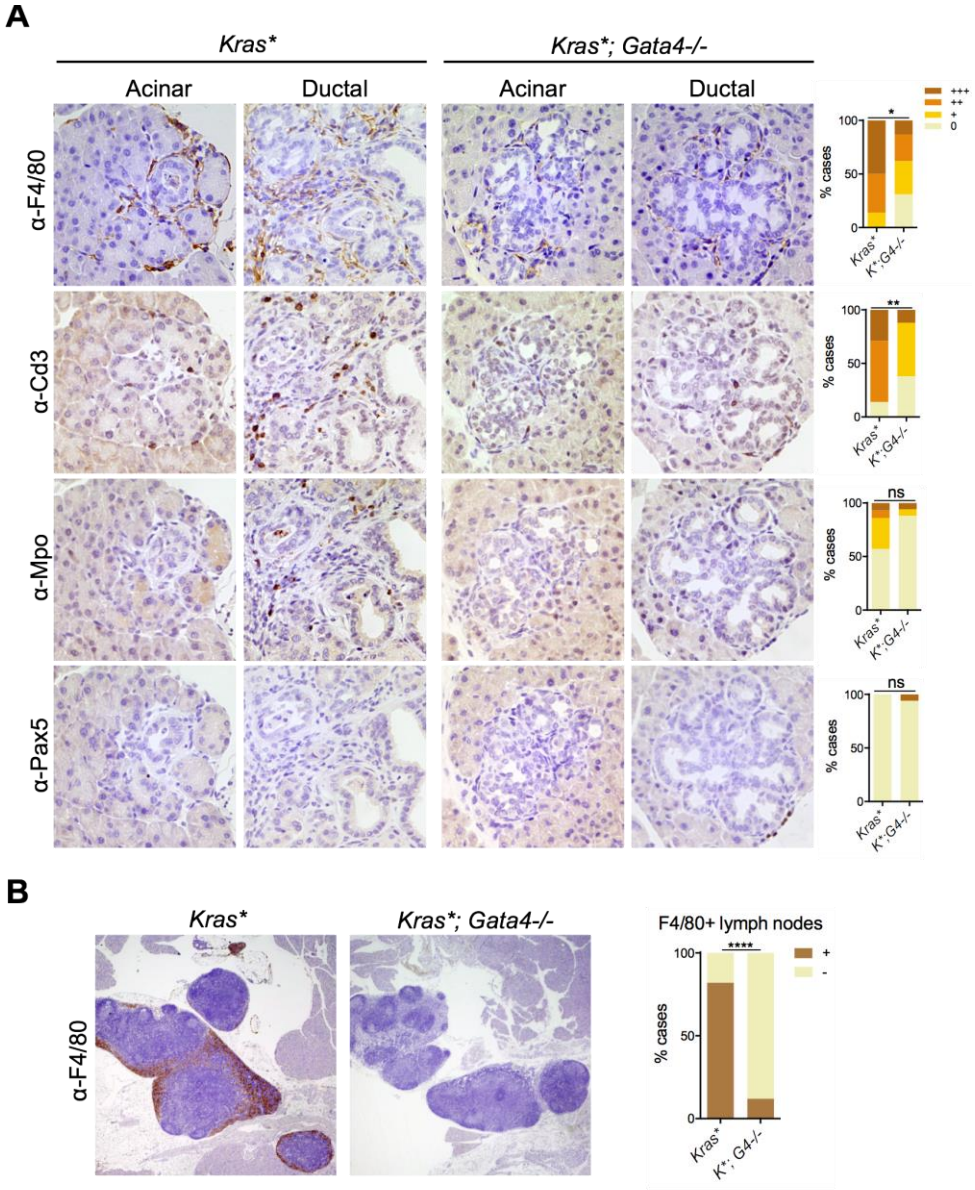

Figure 16. Infiltration of Kras*-induced lesions by macrophages and T lymphocytes is modulated by Gata4. (A) Representative images of IHC and quantification of infiltrating leukocyte populations in the pancreas of 8 week-old *Kras** and *Kras*;Gata4*$^{P-/-}$ (*K*;G4-/-*) mice, including lesions of both acinar and ductal morphology. Quantification is based on number of cells within the lesion: 1-5 cells (+), 6-15 cells (++), >15 cells (+++). Presence of macrophages was quantified according to staining intensity: mild (+), moderate (++), and strong (+++). P<0.05 (*); P<0.01 (**); P<0.001 (***). **(B)** Right panel: Representative images of mesenteric lymph nodes stained for the macrophage marker F4/80 in *Kras** and *Kras*;Gata4*$^{P-/-}$ mice. Left panel: Percentage of F4/80+ lymph nodes in each genotype (P<0.0001).

2.8. Gata4-null primary acinar preparations are defective in macrophage activation

Considering these results, we hypothesized that factors secreted by acinar cells activate macrophage recruitment, which, in turn, leads to infiltration of T lymphocyte in the pancreas. To test this notion, we established suspension cultures of primary acinar cells from control, *Gata4*$^{P-/-}$, *Kras** and *Kras*;Gata4*$^{P-/-}$ mice for 48h in low-serum medium. Conditioned medium was added to RAW264.7 macrophages for 24h and the expression of markers of macrophage activation, such as *Ccl5*, *Cxcl10* and *Cxcl11*, was analysed (Fig. 17). We found that macrophages treated with medium conditioned by Gata4-null acini expressed slightly lower levels of *Ccl5* (P=0.183) and significantly lower levels of *Cxcl10* (P=0.009) and *Cxcl11* (P=0.016) compared to medium from control (*Ptf1a*-Cre$^{+/KI}$) acini.

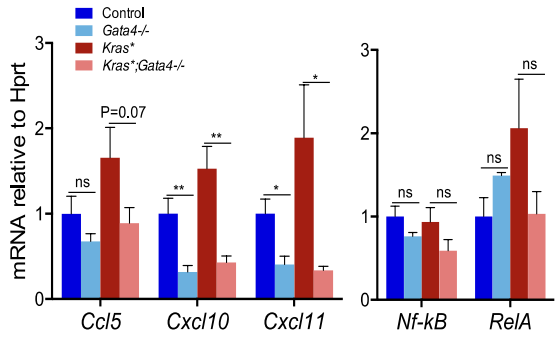

Figure 17. Impaired macrophage activation by conditioned medium from *Gata4*$^{-/-}$ acinar preparations. Conditioned medium (48h) from isolated acinar cells cultured in low-serum medium was added to RAW 264.7 macrophages for 24h. Expression of inflammation activating genes was assessed by RT-qPCR. P>0.05 (ns); P<0.05 (*); P<0.01 (**); P<0.001 (***).

When macrophages were treated with medium conditioned by *Kras** acini, the expression of *Ccl5*, *Cxcl10* and *Cxcl11* was increased respect to *Ptf1a*-Cre controls. Importantly, macrophage treatment with medium conditioned by *Kras*;Gata4*$^{P-/-}$ cells resulted in even lower expression of *Ccl5* (P=0.078), *Cxcl10* (P=0.002) and *Cxcl11* (P=0.028) compared to macrophages treated with medium conditioned by *Kras** acini. We also assessed the ability of the different conditioned media to induce T lymphocyte migration through transwells. Preliminary data showed that conditioned medium from Gata4-null acini does not differentially promote T lymphocyte migration compared to control medium (data not shown), suggesting that the lack of inflammatory response in a Gata4-null context is due to impaired macrophage activation and not due to reduced T lymphocyte recruitment directly from acinar cells.

2.9. Reg3b is downregulated in Gata4$^{-/-}$ pancreata and contributes to macrophage activation

As commented previously, visual inspection of the RNAseq data (Table 1) had highlighted *Reg3b* -and other Reg family members– as some of the most strongly downregulated genes in *Gata4*$^{-/-}$ pancreata (Fig. 18A). Interestingly, *Reg3b* expression is massively induced during pancreatitis (Keim *et al*. 1984). Therefore, we analysed whether loss of Gata4 affected its upregulation. *Reg3b* expression was dramatically upregulated at 24h during caerulein-induced acute pancreatitis in control mice (*Ptf1a*-Cre$^{+/KI}$). By contrast, R*eg3b* levels in *Gata4*$^{-/-}$ pancreata were slightly upregulated, but at much lesser extent compared to controls ($P<0.001$) (Fig. 18B).

A role of Reg3b in neoplastic transformation in the pancreas has been proposed (Loncle *et al*., 2015). Therefore, we examined Reg3b expression in 1-year-old *Kras** mice by IHC. We observed strong expression of Reg3b in acinar cells surrounding areas of metaplasia in the pancreas of *Kras** mice but not in *Kras*;Gata4*$^{P-/-}$ pancreata, not even in areas presenting advanced lesions (Fig. 18C).

It has been reported that Reg3b is necessary to mobilize resident tissue macrophages in an infarcted heart to induce regeneration (Lörchner *et al*., 2015). Since *Gata4*$^{P-/-}$ mice are defective in both pancreatic Reg3b expression and macrophage activation, we hypothesized that Reg3b might be required to activate resident macrophages in the pancreas. To address this hypothesis, we tested the effect of recombinant Reg3b and Reg3b blocking antibodies on the activity of conditioned medium from *Kras** isolated acini using RAW264.7 macrophages as a readout (Fig. 18D). Similar to what we had previously observed, expression of *Mmp9* and *Cxcl16* was lower in macrophages treated with medium conditioned by Gata4-null cells. Induction of these genes was decreased when anti-Reg3b was added in *Kras** medium and, conversely, their expression was increased when recombinant Reg3b was added to *Kras*;Gata4*$^{-/-}$ medium. The differences observed were not statistically significant but the findings suggest a role for Reg3b in macrophage activation.

Gata4 has been proposed to directly regulate *Reg3b* expression in intestinal cells (Lepage *et al*., 2015). To assess whether Gata4 directly also controls *Reg3b* expression in normal pancreas, we examined the presence of peaks in the *Reg3b* genomic locus identified through a Gata4 ChIP-seq experiment generated in our laboratory (data not shown). We did not find a peak at the promoter of *Reg3b*, but we found a Gata4 binding signal in a region in-between all *Reg* family members, 13kB downstream of the *Reg3b* TSS (Fig. 17D). It has been described that *Reg* genes are part of a chromatin loop and that their expression is coordinated; therefore, we hypothesized that the signal observed in the ChIP-seq

data might be part of an enhancer regulating all *Regs* expression. We performed ChIP-qPCR to validate the ChIP-Seq data and found a clear enrichment compared to control IgG (P<0.01) (Fig. 18E). These results suggest that Gata4 regulates *Reg3b* directly in the pancreas.

In order to determine whether the Reg3b production defect might be responsible for the failure of *Gata4*$^{-/-}$ acinar cells to undergo ADM *in vitro*, we analysed the ability of *Reg3b*$^{-/-}$ acinar cells to form cystic structures: *Reg3b*$^{-/-}$ acinar cells displayed an intermediate phenotype, with a moderately reduced ability to undergo ADM, compared to the *Gata4*$^{-/-}$ cells, suggesting that the defect of Gata4-null cells is not only due to low Reg3b secretion (Fig. 18F).

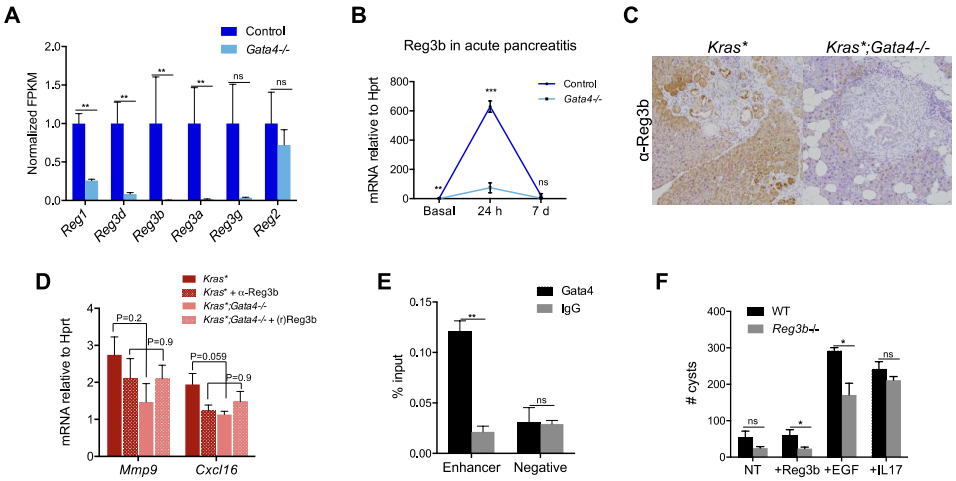

Figure18. Reg3b expression is regulated by Gata4 and contributes to macrophage activation (A) RNA-seq results of expression of *Reg* family members in the pancreas of 8 week-old control and *Gata4*$^{P-/-}$ mice. **(B)** *Reg3b* expression is markedly upregulated upon induction of an acute pancreatitis in control mice but not in *Gata4*$^{P-/-}$ mice. P<0.05 (*); P<0.01 (**); P<0.001 (***). **(C)** Representative IHC images of Reg3b expression in the pancreas of 8 week-old *Kras** and *Kras**;*Gata4*$^{P-/-}$ mice. **(D)** Expression of *Mmp9* and *Cxcl16* in RAW 264.7 cells treated with medium conditioned by (from left to right): **I.** *Kras** acini; **II.** *Kras** acini +α-Reg3b blocking antibody; **III.** *Kras**;*Gata4*$^{-/-}$ acini; **IV.** *Kras**;*Gata4*$^{-/-}$ acini +recombinant (r)Reg3b. **(E)** ChIP-qPCR with anti-Gata4 antibodies to assess binding to a putative enhancer region of *Reg3b*. **(F)** Cyst formation by matrigel-embedded acinar cells from wild type and *Reg3b*$^{-/-}$ mice.

3. ROLE OF GATA4 IN PDAC PROGRESSION AND SURVIVAL

Recent work performed in our laboratory has highlighted the role of Gata6 as a tumour suppressor during pancreatic carcinogenesis. Loss of Gata6 in mouse pancreas accelerates Kras*-driven carcinogenesis (Martinelli *et al*., 2015), and low levels of GATA6 in human PDAC cell lines is associated with enhanced tumour dissemination. In addition, patients with basal-like GATA6-low tumours have a shorter survival (Martinelli *et al*., 2016). All these data led us to investigate the role of Gata4 in an aggressive model of pancreatic carcinogenesis, and GATA4 expression in human samples and how it relates to patient survival.

3.1. *Trp53* inactivation accelerates tumour progression in *Kras*;Gata4*$^{P-/-}$ mice

Multiple genetic events cooperate to promote PDAC development/progression through a variety of molecular mechanisms. *Trp53* is a major gene involved in PDAC progression (Hingorani *et al*., 2005) and we aimed to determine the consequences of inactivating *Trp53* in *Kras** and *Kras**;*Gata4*$^{P-/-}$ mice. We first analysed *Kras**;*Trp53*$^{-/-}$;*Gata4*$^{-/-}$ and control (*Kras**;*Trp53*$^{-/-}$;*Gata4*$^{+/+}$) 6 week-old mice (Fig. 19A). Histological analyses revealed that *Kras** mice presented a low incidence of low-grade PanINs (1/7 cases); in agreement with previous results, no PanINs could be observed in *Kras**;*Gata4*$^{P-/-}$ mice. Although low number of AFL and high-grade PanIN could be observed in both genotypes, the presence of these lesions in a Gata4-null context indicated that Gata4 does not impair their development. Similarly, both mouse strains developed aggressive PDAC with high penetrance, again indicating that Gata4 is not necessary for PDAC development, which are favoured in a P53-null background. We did not observe significant differences in tumour grade, percentage of remodelled area and lipomatosis between genotypes.

Next, we allowed mice age until they had to be sacrificed due to humane endpoint, at which point we analysed the pancreas. We found a low and similar incidence of low-grade PanINs and high-grade PanINs in both mouse strains. Similarly, the rate of areas affected by ADM, the number of AFL, and the occurrence and grade of PDAC were similar. We found a higher prevalence of cystic structures in *Kras**;*Trp53*$^{-/-}$ pancreata, that were absent in *Kras**;*Trp53*$^{-/-}$;*Gata4*$^{-/-}$ counterparts, but the small number of mice analysed precluded statistical analysis (Fig. 19B). Finally, we analysed mouse survival; *Kras**;*Trp53*$^{-/-}$;*Gata4*$^{-/-}$ mice died significantly earlier than controls (P<0.0001) (Fig. 19C). Similar results were obtained when using *Kras**;*Trp53*$^{+/-}$;*Gata4*$^{-/-}$ mice (P=0.0018). Altogether, these results indicate that, Gata4 and P53 cooperate in driving Kras*-initiated PDAC progression and support the role of Gata4 as a bona fide tumour suppressor gene.

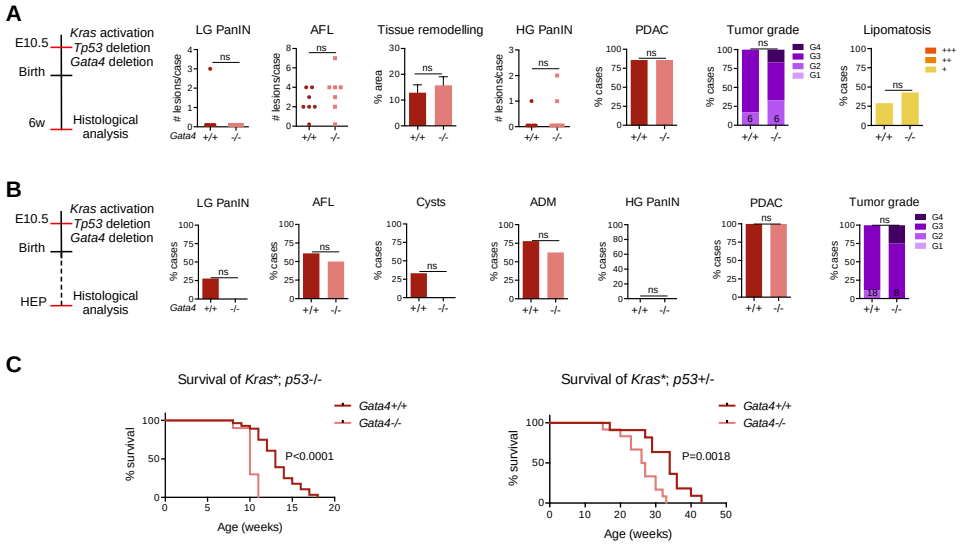

Figure 19. *Gata4* inactivation in *Kras;Trp53*$^{-/-}$ mice accelerates tumour progression. (A)** Experimental design: histology of *Kras**;*Trp53*$^{-/-}$; *Gata4*$^{P-/-}$ mice (in which recombination is mediated by *Ptf1a*-Cre at E10.5) was analysed at 6 weeks of age, before reaching the humane endpoint. Presence of low-grade (LG) PanIN, AFL, high-grade (HG) PanIN, PDAC and its grading, tissue remodelling and lipomatosis was analysed. **(B)** Experimental design: histology of *Kras**;*Trp53*$^{-/-}$;*Gata4*$^{P-/-}$ mice was analysed when reaching the humane endpoint (HEP). Presence of low-grade PanIN, cystic structures, ADM, AFL, high-grade PanIN, PDAC and its grading was analysed. **(C)** Left panel: Survival curve of *Kras**; *Trp53*$^{-/-}$;*Gata4*$^{P-/-}$ (n=10) and control mice (n=28), showing a poorer survival in the absence of Gata4 (P<0.0001). Right panel: Survival curve of *Kras**;*Trp53*$^{+/-}$;*Gata4*$^{P-/-}$ (n=11) and control mice (n=11), again showing a poorer survival in the absence of Gata4 (P<0.0018)

3.2. GATA4-low PDAC displays basal-like phenotype and associates with poor patient survival

To assess the implications of the studies conducted in mice in relationship to the molecular pathogenesis of human PDAC, we analysed GATA4 expression in normal and neoplastic pancreas using several, complementary, experimental strategies.

In normal human pancreas, GATA4 is expressed with a cell-specific pattern similar to that observed in the mouse pancreas: it is detected in acinar cells but not in ductal or endocrine cells (Fig. 20A). We analysed a TMA series of 23 evaluable cases and, interestingly, the majority of low-grade and high-grade PanINs showed detectable levels of GATA4 (out of 27 low-grade PanINs, 1 showed negative, 2 weak, 6 moderate and 18 strong staining intensity; and out of 2 high-grade PanINs, 1 showed moderate and 1 strong intensity). Contrary, expression in tumour cells was much lower (out of 20 PDAC cases, 12 showed negative, and 8 weak staining) (Fig. 20B,C). The differences in staining intensity between PanINs and PDAC was highly significant (P<0.001), but not between

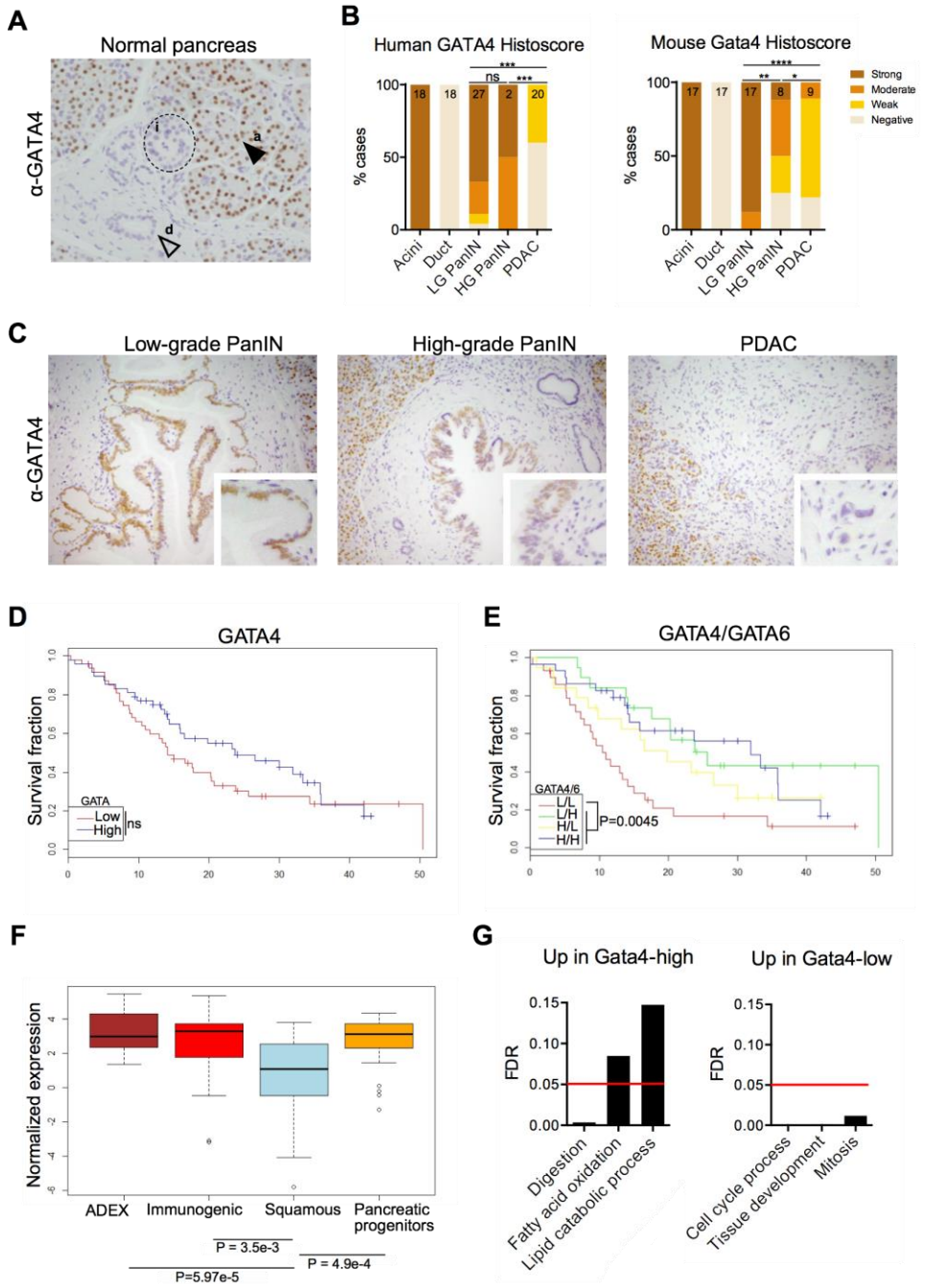

Figure 20. Gata4 loss associates with high-grade lesions, basal-like phenotype, and poor survival in patients with PDAC. (A) Representative image of the expression of GATA4 in normal human pancreas showing expression in acinar cells but not in ductal or endocrine cells. **(B)** Histoscore for IHC analysis of GATA4 expression in the pancreas of patients with PDAC in a series of 23 evaluable cases (left panel), and in 1-year-old *Kras** mice (N=17) (right panel). **(C)** Representative images of the expression of GATA4 in low-grade PanIN, high-grade PanIN, and PDAC. **(D)** Kaplan-Meier plot of survival of patients with high and low GATA4-expressing tumours. Dataset from Bailey *et al*. 2016. **(E)** Kaplan-Meier plot of survival of patients according to the expression of GATA4 and GATA6. Dataset from Bailey *et al.* **(F)** GATA4 expression

(Fig. 20 cont.) according to PDAC subtypes as defined by Bailey *et al*. **(G)** GSEA analysis comparing tumours expressing high vs. low GATA4 mRNA levels (first vs. last quartile). Libraries from Gene Ontology (GO) were used to run the analysis. Significance threshold was set at FDR<0.0.5.

low- and high-grade PanINs (P=0.826). Similar Gata4 expression pattern was observed in neoplastic lesions of *Kras** mice (Fig. 20B).

We took advantage of the transcriptomic data of 456 cases of PDAC published by the Australian ICGC Consortium (P. Bailey *et al*., 2016) and analysed *GATA4* expression. We stratified all cases by considering the median expression of *GATA4* across all cases. We defined tumours with *GATA4* expression higher than the median as "GATA4-high" and those with *GATA4* expression lower than the median as "GATA4-low". Survival analyses showed that patients with GATA4-low tumours had a shorter survival than GATA4-high cases, although the differences were not significant (Fig. 20D). We then surmised that GATA4 and GATA6 might have partially redundant roles in PDAC. A re-analysis of the survival data considering the subgroup of tumours that were GATA6-low/GATA4-low showed a highly significant association with poor outcome in this patient group (P=0.005) (Fig. 20E).

Our laboratory and others have shown that GATA6 is expressed at lower levels in the quasi-mesenchymal or basal-like PDAC subtypes (P. Bailey *et al*., 2016; Martinelli *et al*., 2016). Interestingly, in the Australian ICGC consortium series GATA4 levels were also significantly lower in the squamous subtype (basal-like) than in the other groups (Fig. 20F). These findings are in accordance with reports that PDAC with a basal-squamous phenotype have worse outcome (Bailey *et al*., 2016; Martinelli *et al*., 2016; Moffitt *et al*., 2015).

To get insight into the molecular mechanisms that might participate in this association with survival, we stratified all cases according to GATA4 levels and defined tumours in the top quartile of expression as "GATA4-high" and those in the bottom quartile as "GATA4-low". We performed GSEA applying Gene Ontology (GO) libraries and found that the main pathway enriched in "GATA4-high" was "Digestion", a signature that includes a number of digestive enzymes (Fig. 20G). These results suggest that tumours expressing high Gata4 are more differentiated. Contrary, results from GSEA in "GATA4-low" samples revealed an enrichment in pathways related to enhanced proliferation such as "Cell cycle process", "Tissue development" or "Mitosis" (Fig. 20G).

DISCUSSION

Gata factors play a critical role during early embryo development and during organogenesis. Inactivation of Gata4 or Gata6 at the initiation of pancreatic development does not impede pancreas formation. However, concomitant inactivation of both genes results in pancreas agenesis (Carrasco *et al*., 2012; Xuan *et al*., 2012). Therefore, one Gata member (4 or 6) can compensate the lack of the other to allow pancreas formation, indicating that Gata4 and Gata6 share partially redundant functions during pancreas organogenesis.

Recently, we have reported that Gata6 is crucial for the maintenance of acinar cells in the adult pancreas, as its deletion at e9.5 results in exocrine atrophy, adipocyte transdifferentiation and ADM. These results indicate that Gata6 has specific functions in the adult pancreas that cannot be compensated by Gata4. In addition, we have proposed that Gata6 can act as an important tumour suppressor gene in the pancreas as its deletion in a mutant Kras-induced model of carcinogenesis increases the incidence of PanIN and PDAC (Martinelli *et al*., 2015). In addition, human PDAC cell lines with low GATA6 expression levels display high invasion *in vitro* and enhanced dissemination *in vivo,* and patients with GATA6-low expressing PDAC present poorer survival (Martinelli *et al*., 2016). Given the prominent role that Gata6 plays in pancreas physiology and pathology, and the importance of both Gata4 and Gata6 during pancreas organogenesis, we asked whether Gata4 also plays a critical role in pancreas homeostasis and pathogenesis. Here, I will discuss our results showing that Gata4 has specific functions in the adult pancreas and that it plays a tumour suppressor role in mouse models of PDAC.

1. Gata4 is dispensable for proper exocrine pancreas homeostasis or acinar differentiation

In order to study the role of Gata4 in adult pancreas homeostasis we generated mice lacking Gata4 in the pancreas, as we previously did to study Gata6. We performed a detailed characterization of $Gata4^{-/-}$ pancreata both at the histological and RNA expression level. Unlike Gata6, which is expressed in all exocrine and endocrine cells, Gata4 is only expressed in acinar cells. This result was in accordance with other reports (Decker *et al*., 2006; Ketola *et al*., 2004; Nemer & Nemer, 2003; Ritz-Laser *et al*., 2005). Although we did not observe any Gata4+ endocrine cells, we cannot rule out that Gata4 plays a role in this compartment and that expression levels are under detection capacity of the technique used. Actually, some reports have attributed Gata4 a role in regulating glucagon expression (Ritz-Laser *et al*., 2005) and we have observed that $Gata4^{P-/-}$ mice show some alterations in glucose regulation consisting of lower glycaemia both in basal fasting conditions and upon glucose administration. We investigated possible causes by analysing expression levels of transcription factors that regulate endocrine function and expression of hormones controlling

glycaemia, but we could not detect any significant difference. Since *Gata4*$^{P-/-}$ mice did not have liver or weight problems (data not shown), and the objective of the work was to evaluate the role of Gata4 in exocrine physiology and pathogenesis, we did not investigate this phenotype further and focused on the role of Gata4 in the exocrine pancreas.

The exocrine pancreas of *Gata4*$^{P-/-}$ mice did not show any major histological alterations except for a mild increase in the number of adipocytes and a slightly more basophilic cytoplasm in acinar cells, indicating lower enzyme content. These results were in contrast with those found in *Gata6*$^{-/-}$ pancreas. In addition, proliferation, apoptosis, DNA damage and polarity were not affected in Gata4-null pancreata, unlike in the Gata6-null pancreata. Yet, there were subtle alterations suggesting perturbation of normal differentiation programmes such as the upregulation of Krt19 and a mild downregulation of some digestive enzymes. Thus, although initial studies showing Gata4 expression in the exocrine pancreas attributed it a role in this tissue, our results suggest that it might not be the case under physiological conditions. We therefore conclude that, unlike Gata6 or Ptf1a, which are necessary for the expression of the acinar transcriptome, Gata4 is not a critical driver of acinar gene expression. Indeed, lack of Gata4 results in mRNA upregulation of transcription factors that orchestrate proper acinar differentiation, such as Ptf1a, Gata6 or Nr5a2, suggesting that overexpression of these TF compensates the loss of Gata4.

2. Gata4 as a possible regulator of epithelial-hematopoietic cell crosstalk

Despite the lack of a damaging phenotype in the exocrine pancreas, the RNAseq data highlighted alterations in two pathways in the absence of Gata4: "Hematopoietic cell lineage" and "PPAR signalling". Hematopoietic cells can be grossly classified as being involved in the "innate" or "adaptive" immune response. Further analysis of the RNAseq data revealed that in *Gata4*$^{-/-}$ pancreas there is a significant downregulation of pathways controlling innate, but not adaptive, immunity (Fig. D1A).

Innate immune cells (Fig. D1B) have been classically viewed as the responsible of eliciting an inflammatory response. Initially, cells of the innate immunity were described to trigger inflammation by recognizing foreign microbial and viral structures, (pathogen-associated molecular patterns, PAMPs). Studies in the last years have also attributed them the ability to induce inflammation upon recognition of normal cellular constituents released during injury and cell death (damage-associated molecular patterns, DAMPs) (Fig. D1C). Both PAMPs and DAMPs are recognized by pattern-recognition receptors (PRRs), which induce innate immune cells to upregulate co-stimulatory molecules as well as numerous

inflammatory chemokines and cytokines that attract and prime other leukocytes for activation, thus triggering inflammation (Shalapour & Karin, 2015).

Figure D1. Leukocytes in damage and steady state of the pancreas. (A) GSEA results showing decreased regulation of pathways related to innate (but not adaptive) immunity in *Gata4*$^{-/-}$ pancreas compared to control. (B) Schematic representation of innate and adaptive immune cells. Hematopoietic Stem Cells (HSC) give rise to the Common Myeloid and Lymphoid Progenitors (CMP and LMP, respectively). The CMP gives rise to neutrophils (and other granulocytes) and to monocytes, which can differentiate into macrophages and a subset of dendritic cells (DCs). Together with other leukocytes (i.e. NK cells), they comprise the innate immunity cells. The LMP gives rise to B and T lymphocytes, as well as a subset of DCs, constituting the adaptive immunity. DCs are antigen-presenting cells highly specialized in triggering an adaptive immune response, and therefore they are considered the link between innate and adaptive immunity. *Adapted from Rieger & Schroeder, 2012*. (C) Basic representation of sterile (pathogen-free) inflammation. Upon injury, damaged cells release DAMPs that activate resident macrophages to induce inflammation and recruit neutrophils and monocytes that differentiate mainly into macrophages. Both of them remove dead cells, and then macrophages secrete anti-inflammatory cytokines that resolve inflammation and factors that promote tissue regeneration. Although not represented for simplification, the inflammatory process also requires a number of additional leukocytes that orchestrate the response, specially T lymphocytes. (D) RNAseq data showing expression of markers for leukocytes (*Ptprc,* coding for Cd45, P=0.006); myeloid cells (*Cd11b*), including macrophages (*Adgre1, Mrc1, Clec10a*, coding for F4/80, Cd206 and Cd301, respectively); DCs (*Zbtb46,* P=0.04); B lymphocytes (*Cd19, Pax5*); and T lymphocytes (*Cd3d, Cd4, Cd8*); in control (Gata4+) and Gata4-null (-) pancreata. Distribution of islet and exocrine leukocytes described in Calderon *et al*., 2015 is also shown.

Innate immune cells not only sense the damage and induce inflammation, but also remove dead cells and promote tissue regeneration. Neutrophils and macrophages are responsible for phagocytosis and clearance of cellular debris.

While the first are specialized in degrading completely the phagocytised particle, the latter are able to present phagocytised antigens to regulate inflammation towards resolution and promote tissue recovery (Fadok *et al*., 1998; Gea-Sorlí & Closa, 2010; Ortega-Gómez *et al*., 2013) (Fig. D1C).

It has been shown that leukocyte subpopulations display distinct distribution within the pancreas in basal conditions. Thus, macrophages predominate in the islets of Langerhans (>98%), while the exocrine pancreas contains a mixture of myeloid cells (mainly macrophages), DCs, and B and T lymphocytes (Fig. D1D) (Calderon *et al*., 2015). This study shows how macrophage depletion and replacement by donor stem cells results in a precise reconstitution of cellular profiles. Thus, maturation of each macrophage subpopulation depends on specific factors, as mice lacking functional *Csf1* (Macrophage-Colony Stimulating Factor, M-CSF) showed a profound decrease in "islet" but not "exocrine" macrophages. Altogether, these results indicate the existence of an active epithelial-hematopoieti communication in the pancreas even in the absence of injury.

Another study showed that mice lacking Major Histocompatibility Complex class II (MHC-II) expression developed a selective, progressive and fatal loss of exocrine, but not endocrine, pancreatic cells. Histological analyses revealed a progressive lymphocytic cell infiltration as the cause of the damage (Vallance *et al*. 1998). Interestingly, MHC-II is expressed by APCs to avuid autoimmune diseases, among other functions (Unanue *et al*., 2016). Consequently, a defect in this pathway might be deleterious for tissues with high self-antigen presenting activity. Since the pancreas is loaded with huge amounts of proteases (a potential weapon of mass destruction if released uncontrolledly), we speculate that a high self-antigen presentation rate would allow a rapid detection of any alteration and an effective repair by the immune system. This putative high rate of self-antigen presentation in the pancreas would explain the variety of leukocytes populating the exocrine, but not the endocrine, pancreas (Calderon *et al*., 2015); and again highlights the importance of tissue-specific communications between the epithelium and the immune system.

A role of Gata4 in epithelial-hematopoietic crosstalk is also suggested by a significant downregulation of *Ptprc* (Cd45) in *Gata4*$^{-/-}$ pancreas compared to control. Markers of myeloid cells and DCs are also consistently downregulated; while lymphoid markers are barely detected, except for the B cell marker CD19, which regulates B cell responses to self-antigens (Fearon & Carroll, 2000), and is also downregulated in *Gata4*$^{-/-}$ pancreata (Fig. D1D).

The other significantly downregulated pahtway in *Gata4*$^{-/-}$ pancreas was "PPAR signalling" (Fig. 3B). PPAR signalling has been shown to be necessary to maintain an M2 (anti-inflammatory) phenotype in resident macrophages in other tissues

(Kang *et al*., 2008; Odegaard *et al*., 2007), a feature that is also observed in pancreatic exocrine macrophages (Calderon *et al*., 2015). Since markers of M2 macrophages also appear downregulated in our RNAseq data in the absence of Gata4 (not shown), low PPAR signalling in *Gata4*$^{-/-}$ pancreata might be indicative of a defect in macrophage polarization. Nevertheless, the decrease in PPAR signalling could also be related to lipid metabolism, as *Gata4*$^{-/-}$ pancreata show a slight increase in lipomatosis in basal conditions.

Among all leukocyte markers we checked in our RNAseq data (Fig. 15A), macrophages were the only ones decreased in Gata4-null pancreas independently of *Kras* status (WT or mutant); while markers of other subpopulations were similarly expressed in all conditions except in Kras* -where they increase-, evidencing pancreas immune cell infiltration upon oncogenic signalling. In a *Kras** background, the decrease of all immune cell markers in a Gata4-null context is also indicative of a role for Gata4 in regulating innate immune cells.

Last, but not least, an unbiased genome-wide study identified GATA factors as the main regulators of epithelial innate immune responses in *C. elegans.* In addition, the authors proposed that this function is conserved in humans, as GATA6 was found to have a protective function in lung epithelial cells exposed to *P. aeruginosa* (Shapira *et al*., 2006).

Altogether, these notions suggest that Gata4 regulates a crosstalk between epithelial and hematopoietic ells in the exocrine pancreas by inducing expression of genes necessary for proper homeostasis of resident immune cells. This idea allows interpreting some of the results presented in this work, and may be useful for further research on the field.

3. Gata4 phosphorylation during acinar stress is indicative of an enhanced transcriptional activity

The role of Gata4 during heart development, cardiomyocyte differentiation and stress responsiveness has been a matter of intense research (Borok & Papaioannou, 2016; Garg *et al*., 2003; Han *et al*., 2012; Holtzinger & Evans, 2005; Hu *et al*., 2011; Katanasaka *et al*., 2016; Kuo *et al*., 1997; Li *et al*., 2012; Liang *et al*., 2001; Mehta *et al*., 2015; Molkentin *et al*., 1997; Oka *et al*., 2006; Pu *et al*., 2004; Van Berlo *et al*., 2011; Watt *et al*., 2004; Yu *et al*., 2016; Zhao *et al*., 2008; Zhou *et al*., 2012), providing insight into the regulation of its activity (Table D1). Gata4 post-translational modifications have been shown to enhance its transcriptional activity in response to hypertrophic stimuli. Specifically, phosphorylation of serine 105 mediated by Mek1-Erk1/2 kinases (Liang *et al*., 2001) is one of the most common Gata4 post-translational modifications, and is

necessary for stress-induced cardiac hypertrophy *in vivo* (van Berlo *et al*., 2011).

Modification	Residue	Function	Mechanism	Enzyme
Acetylation	K312/319/ 321/323	Transcriptional activation	Increased DNA binding and transcriptional activity	p300
Phosphorylation	S105	Transcriptional activation	Increased DNA binding	ERK1/2, p38
	S160	Transcriptional activation	Increased interaction with co-activators	CDK4
	Unknown	Transcriptional inhibition, nuclear export of Gata4	Increased interaction with Crm1	GSK3-β
	S261	Transcriptional activation	Increased acetylation by p300	ERK1/2, RSK2, PKA
	S419/420	Transcriptional activation	Increased DNA binding activity	PKC
Methylation	K299	Transcriptional activation	Decreased interaction with p300	EZH2
SUMOylation	K366	Transcriptional activation	Facilitation of nuclear localization	SUMO-1, PIAS1

Table D1. Post-translational modifications that modulate Gata4 transcriptional activity. *Adapted from Katanasaka, et al., 2016*

We hypothesized that stress conditions might also induce Gata4 phosphorylation in the pancreas. Phopho-S105 was up-regulated in acinar cells in multiple experimental settings that trigger MAPK (Mek1-Erk1/2) activation, including acinar cell isolation and culture (Pinho *et al*., 2011), pancreatitis (Halbrook *et al*., 2017), and mutant Kras-induced PanIN development (Collins *et al*., 2014). It is conceivable that pS105-Gata4 activates a distinct set of genes that are not expressed under physiological conditions that allow acinar cells to deal with stress/damage. For instance, Reg3b plays a role in pancreatitis as a mitogenic and anti-apoptotic factor to enhance tissue regeneration after damage (Gironella *et al*., 2013; Simon *et al*., 2003), and we have found that its induction during pancreatitis is directly regulated by Gata4.

Since Gata4 is activated upon damage and its loss does not greatly affect exocrine pancreas homeostasis, it is possible that the major Gata4 function is related to pathological situations. To test this hypothesis, we induced a mild cerulein-induced pancreatitis in Gata4-null mice. Some damage indicators (oedema, acute inflammation, acinar vacuolization) were consistently increased in the absence of Gata4, suggesting that Gata4 participates in the regenerative processes.

However, Gata4 was not absolutely required for leukocyte homing or for complete tissue recovery, indicating that other factors and signalling pathways compensated for its absence.

In a model of induced intracellular activation of trypsinogen, this event is sufficient to cause massive cell death, which generates DAMPs that trigger inflammation (Gaiser *et al*., 2011), a process that is also observed in cerulein-induced pancreatitis, yet to a lesser extent (Hofbauer *et al*., 1998). As previously commented, the contents of dead cells released to the interstitium are sensed by innate immune cells as damage signals, triggering inflammation (Piccinini & Midwood, 2010). Then, it is possible that pancreatitis in control and *Gata4*$^{P-/-}$ mice develops in a similar manner as it is likely triggered by DAMPs in both genotypes, independently of Gata4.

4. Gata4 is required for PanIN but not for PDAC development

Mutant *Kras* activation in the pancreas also induced Gata4 phosphorylation, as expected from the fact that Kras* activates Erk1/2 (Liang *et al*., 2001). Therefore, we hypothesized that Gata4 might be relevant to mediate Kras* oncogenic signalling. To test this idea, we studied Kras*-induced pancreatic tumourigenesis in a model where *Gata4* deletion occurs concomitantly to *Kras** activation early during pancreas organogenesis. In the absence of Gata4 there was a dramatic decrease in metaplastic ductal lesions, both tubular complexes (TCs) and PanINs, and in some cases the ductal-reprogrammed area was completely absent. Nevertheless, PDAC developed at a frequency similar to that of *Kras** mice. In the light of these results, we propose that:

1) Kras* signals through Gata4 to initiate ADM resulting in TCs and PanIN formation, possibly through phosphorylation by Erk1/2, which induces the activation of Gata4 target genes leading to phenotypic changes.

2) Gata4 is not required for cells to acquire a ductal-like phenotype, defined by expression of Krt19 and occurrence of a glandular lumen. Although PanIN and PDAC are both ductal structures, their architectural differences rely not only in cell-autonomous features (mutations), but also in non cell-autonomous (micro-environment). For instance, in the *Kras** model, PanIN-associated macrophages present a pro-inflammatory M1 phenotype, while in later stages of carcinogenesis they switch to an anti-inflammatory M2 phenotype, which drives pancreatic fibrogenesis and supports tumourigenesis (Liou *et al*., 2017). Preliminary observations suggest that Gata4-null and *Gata4*$^{+/+}$ PDAC stroma contain similar leukocyte subpopulations (data not shown). Thus, leukocytes in *Gata4*$^{-/-}$ PDAC might be contributing to the ductal phenotype through secretion of specific cytokines (i.e. IL17).

3) PDAC can arise in the absence of PanIN. This is a notable contribution of this work, as it allows dissecting the mechanisms leading to PanIN and PDAC. Co-occurrence of both lesions in nearly all carcinogenesis models used to date preculded studying them as separate events. Only one model has also shown PanIN-less PDAC, yet it is based on expression of *Kras** and mutant *Trp53* by ductal cells (Bailey *et al*., 2015). As growing evidence suggest that PanINs arise mainly from acinar cells, this model might not be directly relevant for the study of PanIN-PDAC relationship.

The classical model of tumour progression states that PDAC arises from PanIN through the accumulation of mutations and increase of atypia, yet this idea does not fit in our model. Recently, atypical flat lesions (AFL) have been postulated to be PDAC precursors as well (Aichler *et al*., 2012), a fact that questions the canonical progression model. Although histopathological analysis revealed that classical AFL were nearly absent in *Kras*;Gata4*$^{-/-}$ pancreas, other lesions resembling AFL were observed (AFL-like lesions), suggesting the existence of a wide array of morphologically distinct, but related, PDAC precursor lesions. One histological difference between these lesions relates to the stromal response, which is abundant in AFL (Fig. D2A) and scarce in AFL-like lesions (Fig. D2B).

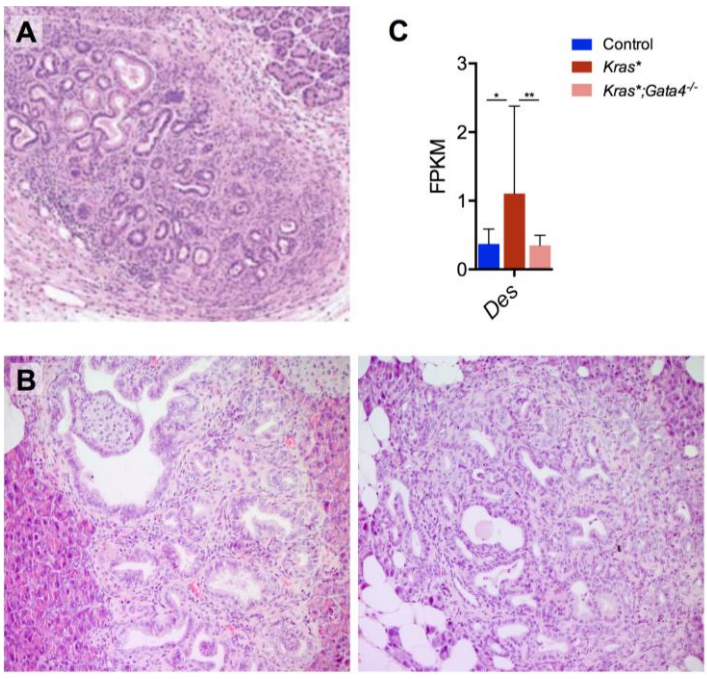

Figure D2. AFL-like lesions. **(A)** Classical atypical flat lesion (AFL), showing a dense stromal response (*extracted from Aichler et al., 2012).* **(B)** Representative microphotographs of AFL-like lesions from *Kras*; Gata4*$^{P-/-}$ pancreata showing a less fibrotic and highly cellular stroma. **(C)** Expression of the PSC marker Desmin (*Des*) showing an increase in Kras* compared to control and *Kras*; Gata4*$^{-/-}$ pancreas. P>0.05 (ns); P<0.05 (*); P<0.01 (**); P<0.001 (***).

PSCs, which are responsible for extracellular matrix deposition, have been shown to differentiate from monocytes during pancreatic regeneration (Ino *et*

al., 2014). On the basis of our hypothesis proposing a role of Gata4 regulating cells of the innate immunity, loss of Gata4 might result in reduced monocyte infiltration and PSC differentiation, and reduced stromal response. Our RNAseq data shows a significant increase of the PSC marker Desmin compared to both control and *Kras*;Gata4*$^{P-/-}$ pancreas (Fig. D2C), indicating that Kras*-induced inflammation is accompanied by increased presence (and probably activation) of PSCs, which is halted in the absence of Gata4.

It has been reported that the transcription factor Sox9 is also necessary for PanIN development from acinar cells -and not centroacinar or ductal cells (Kopp *et al*., 2012)-, which is in accordance to our results. The authors claimed that Sox9-dependent expression of genes necessary for ductal reprogramming during PanIN formation are the principal mechanism for PDAC initiation, as their analysis of 1 year-old *Sox9*$^{P-/-}$ mice did not reveal PanINs or PDAC. However, Gata4 and Sox9 likely orchestrate different pathways, as Sox9 is required to establish a ductal phenotype whereas our data suggest that Gata4 is necessary for Kras*-induced ADM and PanIN formation, but not for acquiring a ductal phenotype itself. This notion is evidenced by the different experiments presented in this thesis showing that *Gata4*$^{-/-}$ acinar cells can actually acquire a ductal phenotype (pancreatitis in *Gata4*$^{P-/-}$ mice [Fig. 6B]; IL17 treatment *in vitro* and *in vivo*, [Fig. 11, 12]; and PDAC formation, [Fig. 7, 9]).

The incidence of PanIN/PDAC discussed above is based on experiments in which *Gata4* deletion and *Kras** activation takes place during embryo development. Gata4 has been reported to play a role in pancreas specification and its deletion during organogenesis might have deleterious effects in the adult tissue. Therefore, we deleted *Gata4* and activated *Kras** selectively in adult acinar cells using the *Ptf1a*$^{CreERT2/+}$ strain by inducing recombination at 8 weeks of age, when Ptf1a is only expressed in acinar cells (Pan *et al*., 2013). PDAC arising in this context unequivocally originated from acinar cells, as shown by the detection of YFP from the *Rosa26*$^{YFP/YFP}$ allele. Importantly, the frequency of tumours observed in *Kras*Gata4*$^{P-/-}$ mice was not significantly different compared to *Kras** mice, indicating that, in adult mice, chronic pancreatitis can promote pancreatic carcinogenesis independently of Gata4. Moreover, these experiments support other studies showing an acinar origin of PDAC.

These results also support a non-linear progression model where PDAC does not necessarily arise from low-grade PanIN. This hypothetical alternative model, which has been already postulated (Real *et al*., 2008), implicates that mutant Kras drives low-grade PanIN in parallel to PDAC, but that these two pathways are not necessarily linked at the cellular level and do not represent sequential events.

5. IL17 induces ADM *in vitro,* but not PanIN *in vivo*, in *Gata4*$^{-/-}$ acinar cells

In order to determine whether *Gata4*$^{-/-}$ acinar cells can undergo ADM *in vitro*, we established 3D cultures and treated them with cytokines that were induced in *Kras** pancreas (compared to control in RNAseq data), as well as other cytokines known to induce ADM. EGF and IL17 were the only factors that consistently induced ADM in *Gata4*$^{+/+}$ acini. Both of them also enhanced ADM in *Gata4*$^{-/-}$ acini, but IL17 was most active in this assay. Importantly, EFG-treated *Gata4*$^{-/-}$ cells lost viability unlike EGF-treated *Gata4*$^{+/+}$ cysts, suggesting a crucial role of Gata4 in Egfr signal transduction, possibly through the Ras-Erk pathway. Contrarily, IL17 signalling induces ADM in a manner independent from Gata4 (Fig. D3). Yet, further work is needed to substantiate this hypothesis.

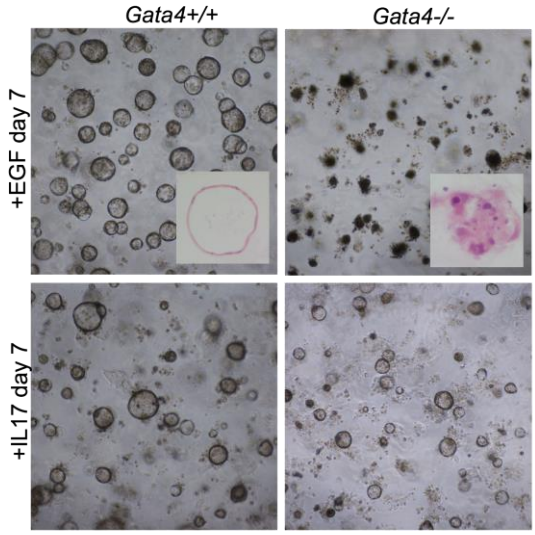

Figure D3. ADM induced by EGF, but not IL17, requires Gata4. 3D cultures of *Kras*^{WT} acinar cells were analysed at late time points (day 7). While EGF-treated *Gata4*$^{+/+}$ cells still present a cystic structure, *Gata4*$^{-/-}$ cells undergo collapse. In contrast, IL17 promotes ADM until late time points in both genotypes, suggesting that EGF and IL17 activate different pathways to induce ADM.

In vivo, IL17 has been shown to induce PanIN development in *Kras** mice (Loncle *et al*., 2015; Mcallister *et al*., 2014). We have reproduced these results showing a higher prevalence of PanINs in adeno-IL17-injected than in adeno-GFP-injected *Kras** mice. Contrarily, *Kras*;Gata4*$^{P-/-}$ mice receiving adeno-IL17 showed a sharp increase in lipomatosis and mucin-negative metaplasia, but not PanINs.

Altogether, these results suggest that:

1. Gata4 is downstream of the Egfr-Kras-Erk pathway and mediates a cell-autonomous response that leads to ADM in a *Gata4*$^{+/+}$ context (cysts *in vitro*, PanINs *in vivo*), and to cyst collapse in *Gata4*$^{-/-}$ (failure in maintaining a cystic phenotype *in vitro,* lack of PanIN *in vivo*). These results are in accordance with the fact that both EGF and *Kras** signal through the MEK-ERK1/2 pathway, which phosphorylates Gata4, thus modulating its transcriptional activity.

2. Gata4 is required for acquiring a PanIN phenotype. We have shown that IL17 promotes abnormal metaplasia instead of PanINs in a Gata4-null context. IL17 is a pro-inflammatory cytokine that promotes both immune cell responses (Jin & Dong, 2013; Jovanovic *et al*., 1998) and tumour growth, enhancing cell survival (L. Wang *et al*., 2009), and inhibiting apoptosis (Nam *et al*., 2008). Although the specific mechanisms through which IL17 induces metaplasia in *Gata4*$^{-/-}$ pancreas need to be further elucidated, we demonstrate that Gata4 is necessary for developing a PanIN phenotype.

3. IL17 promotes ADM through mechanisms different from EGF, the former acting through Gata4-independent pathways and the latter through cell-autonomous mechanisms involving Erk-Gata4 axis. Indeed, IL17 has been reported to signal through the Stat3 pathway to induce ADM (Loncle *et al*., 2015; Mcallister *et al*., 2014). Interestingly, we observed no phospho-Stat3 activation in *Gata4*$^{-/-}$ PALes (Fig. 13), while we could observe phospho-Stat3 positive acinar cells upon IL17 treatment *in vivo* (not shown). These observations suggest that ADM can result from different stimuli (EGF or IL17 treatment) through activation of different mechanisms (Kras-Erk-Gata4 and phospho-Stat3, respectively).

6. A possible mechanism by which Gata4 regulates PanIN differentiation

A significant increase in lipomatosis is observed in *Kras**;*Gata4*$^{-/-}$ pancreata. Similar results have been observed in mouse models where acinar cells acquire an incomplete differentiation due to deletion of transcription factors controlling pancreatic organogenesis. Specifically, lineage tracing experiments have shown that inactivation of c-Myc or Gata6 during embryonic development results in replacement of immature acinar cells by adipocytes (Bonal *et al*., 2009; Martinelli *et al*., 2012), in part through transdifferentiation. Similarly, E2F transcription factors (E2F1/E2F2), which are ubiquitous key regulators of cell growth control, are necessary to maintain differentiation in pancreatic cells, and their deletion causes pancreas atrophy and replacement by ductal structures and adipose tissue (Iglesias *et al*., 2004). These studies suggest that incomplete acinar cell differentiation can favour transdifferentiation into adipocytes. Since Gata4 is required for PanIN development, its absence might promote adypocyte transdifferentiation in PanIN-to-be cells. The sharp increase of lipomatosis and the lack of PanINs in *Kras*;Gata4*$^{P-/-}$ mice infected with adeno-IL17 supports this idea.

We think that Gata4-dependent PanIN development is related to the requirement of both Gata4 and Shh to regulate a gastric phenotype, which characterize PanINs. The evidences supporting this idea are listed below:

1. Hedgehog proteins (Shh, Ihh) play a critical role in early patterning of the primitive gut, from which the pancreas and the stomach develop. Both stomach specification and adult gastric gland differentiation depend on the Hedgehog pathway (Van den Brink, 2007; Van den Brink *et al*., 2001). Similarly, Gata4 plays a crucial role in gastric differentiation: by E18.5, *Gata4*$^{-/-}$ cells fail to support terminal differentiation of the gastric lineages. Importantly, lack of similar phenotypes in mice deficient for Gata5 or Gata6 indicate a unique role for Gata4 in regulating differentiation of definitive endoderm to the stomach's glandular epithelium (Jacobsen *et al*., 2002).

2. PanINs are structures with a characteristic morphology and gene expression that is not typically observed in the normal pancreas. As described previously, PanINs express mucins and other proteins that are not expressed by either ductal or acinar cells. In order to determine the expression profile of PanINs, a study analysed the gene expression profile of microdissected PanINs compared to pancreatic ductal cells. It was found that PanINs activate expression of foregut markers, especially genes that are expressed during gastric differentiation. In addition, it was proposed that this expression switch was mediated through activation of Hedgehog pathway (Prasad *et al*., 2005), which is needed not only for PanIN development, but also for progression to pancreatic cancer (Thayer *et al*., 2003).

3. The role of both Shh and Gata4 in gastric differentiation and PanIN formation suggests an interplay between the two factors during stomach and PanIN development. Interestingly, Gata4 and Gata6 bind to a *Shh* enhancer and regulate its expression during pancreas organogenesis (Xuan & Sussel, 2016). We therefore hypothesize that Gata4 regulates Shh expression to induce PanIN transdifferentiation. Preliminary results show that control and *Gata4*$^{-/-}$ pancreata express low levels of *Gli1* and *Gli2* (effectors of Shh signalling), while their expression increase in *Kras** but remains low in *Kras**;*Gata4*$^{-/-}$ pancreas; and a similar expression pattern is observed for *Shh* (Fig. D4A).

In addition, IHC analysis of Gli1 shows higher expression in PanINs compared to PALes (Fig. D4B). Altogether, these results suggest that Gata4 contributes to Shh regulation during PanIN formation. Yet, we have also observed Gli1 expression in tumours independently of Gata4 (Fig. D4B), supporting the idea that Shh is necessary for PDAC development cancer (Thayer *et al*., 2003), and that is expressed in a Gata4-independent manner at late stages of carcinogenesis.

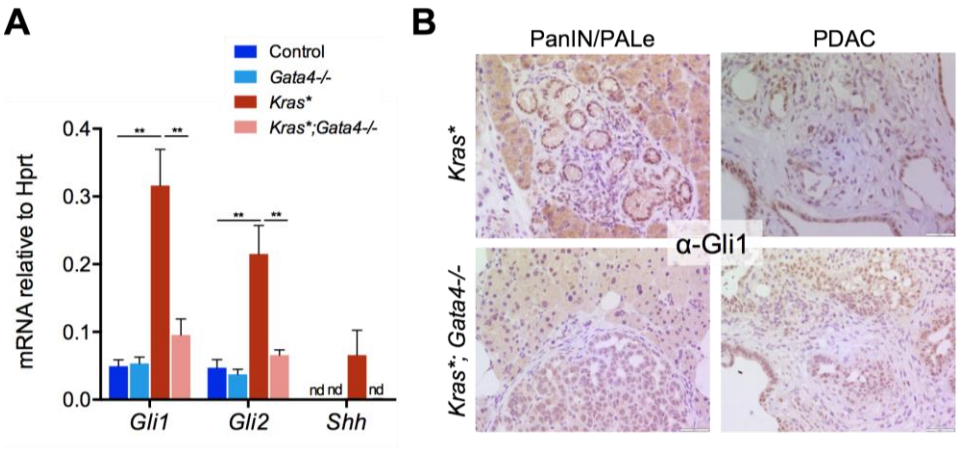

Figure D4. Gata4 regulates Hedgehog signalling. (A) mRNA expression showing an upregulation of *Gli1*, *Gli2* and *Shh* in Kras* pancreata, which is blunted in *Kras*;Gata4*$^{-/-}$. P<0.05 (*); P<0.01 (**); P<0.001 (***); nd (not detected). **(B)** Gli1 expression in PanIN, PALe and PDAC.

7. Gata4 mediates Kras-induced inflammation

The results from the RNAseq experiment show that Kras* induces expression of mediators that attract leukocytes and promote inflammation, and that this response is blunted in Gata4-null pancreata. Generally, tissue resident macrophages are the main responsible of initiating an inflammatory response (Davies *et al*., 2013), and likely play a role in Kras*-induced inflammation. Accordingly, macrophages are the major leukocyte subpopulation present in ADM and PanINs (Clark *et al*., 2007) and, importantly, it has been shown that in response to Kras*, acinar cells express Icam1 that activates macrophages to elicit an inflammatory response (Liou *et al*., 2015).

We hypothesized that Gata4 regulates expression of genes necessary to stimulate macrophages upon Kras activation. To explore this notion, we isolated acinar cells and showed that multiple factors involved in macrophage activation are downregulated in Gata4-null acini, including Reg3b (Lörchner *et al*., 2015) and the Cxr4-Ccl12 axis (Lee *et al*. 2003), which is also known to be important during pancreatic cancer progression (Wu *et al*., 2013) through the activation of Sonic Hedgehog pathway (Singh *et al*., 2012).

Although the specific acinar-secreted factors that activate macrophages need to be further characterized, our data show that medium conditioned by *Gata4*$^{-/-}$ acini is less effective in inducing cytokine expression by cultured macrophages. Although Reg3b is involved in macrophage activation, other known (i.e. Cxcl12) and unknown factors regulated by Gata4 are likely playing a role as well. The analysis of the secretome of *Kras** and *Kras*;Gata4*$^{-/-}$ acinar cells will allow dissecting the factors that are differentially expressed and may play a role in macrophage activation.

8. PanINs constitute a tumour suppression barrier induced by inflammation and oncogenic stress.

A role of inflammation in PanIN development has been described at the molecular and cellular levels. At the molecular level, cytokines such as IL17, TNF-α and Ccl5 are known to induce PanIN development (Liou *et al*., 2013; Mcallister *et al*., 2014). At the cellular level, macrophages play a pivotal role in secreting cytokines and expressing metalloproteases that allow PanIN formation and extracellular matrix remodelling (Liou *et al*., 2013), and, as already described, acinar cells express Icam1 upon Kras* activation that attracts macrophages to promote PanIN development (Liou *et al*., 2015).

PanIN-1 are known to be senescent structures (Caldwell *et al*., 2012; Collado *et al*., 2005). The senescence program locks the cells into a cell-cycle arrest that prevents the spread of damage to the next cell generation and precludes potential malignant transformation. A plethora of stresses can provoke cellular senescence, including telomeric dysfunction resulting from repeated cell division (replicative senescence), mitochondrial deterioration, oxidative stress, severe DNA damage and chromatin disruption (genotoxic stress), as well as the expression of certain oncogenes (oncogene-induced senescence) (Copp *et al*., 2010). However, the senescent phenotype is not limited to an arrest of cell proliferation. In fact, senescent cells are metabolically active and undergo profound changes in protein expression and secretion, ultimately acquiring a Senescence-Associated Secretory Phenotype (SASP). SASP comprises interleukins, inflammatory cytokines, and growth factors that affect surrounding cells, reinforcing senescence, activating immune surveillance and promoting cell survival (Acosta *et al*., 2013).

Considering that PanINs are Kras*-induced senescent structures, they likely contribute to stop oncogenic transformation. In a *Kras*^{WT} context, damage-induced inflammation promotes dedifferentiation of acinar cells, both being required for pancreas regeneration (Fig. D5A). However, when inflammation is not resolved due to continuous Kras signalling, cells cannot undergo acinar re-differentiation (Morris *et al*., 2010) and, since inflammation is not resolved, epithelial cells continue receiving pro-survival signals. Since dedifferentiated cells have more potential to become tumourigenic (Friedmann-Morvinski *et al*., 2012; Hermann *et al*., 2014; Kumar *et al*., 2012; Martinelli *et al*., 2015; Ziegler *et al*., 2013), the continuous proliferative and pro-survival stimuli might trigger the induction of protective mechanisms such as senescence, thus leading to PanIN development (Fig. D5B). Therefore, PanINs may be considered as a protective mechanism -in which dedifferentiated cells susceptible of oncogenic transformation undergo senescence- and not as direct PDAC precursors.

The tumour-limiting effect of PanINs during oncogenic transformation has been

proposed in a number of studies. For instance, the study of Toll-like Receptors (TLRs), which bind DAMPs to trigger inflammation, has highlighted the importance of developing a proper inflammatory response in order to induce PanIN and limit carcinogenesis. Binding of DAMPs to TLRs results in an inflammatory response through Myd88 signalling pathway. Concretely, TLR9 stimulation induces PSCs to become fibrogenic and secrete chemokines that promote inflammation, epithelial cell proliferation and PanIN development (Zambirinis *et al.*, 2015); and similar results have been found for TLR7 (Ochi *et al.*, 2012a) and for TLR4 (Ochi, Nguyen, *et al.*, 2012b). Puzzlingly, inhibition of the TLR signalling transducer Myd88 results in exacerbated pancreatic inflammation and enhanced malignant progression due to a defect in DCs function (Ochi *et al.*, 2012b).

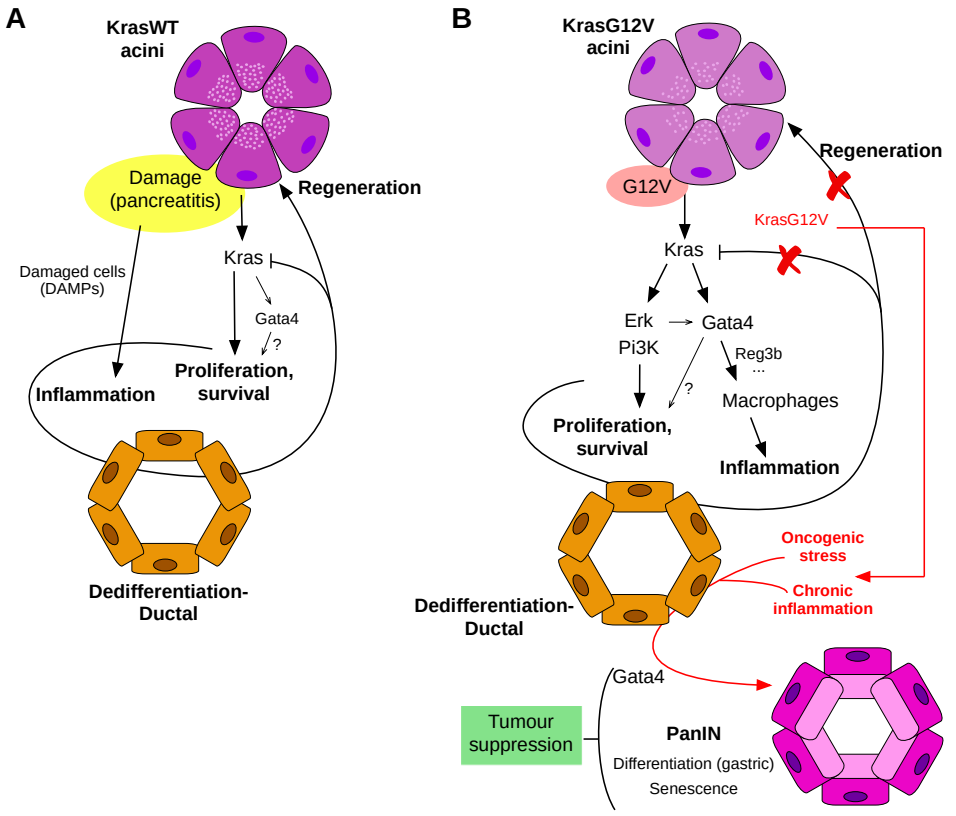

Figure D5. PanINs are tumour suppression barriers. (A) In Kras^{WT} context, tissue damage (pancreatitis) induces DAMP-mediated inflammation and activates Kras to promote cell survival. These signals induce acinar cell dedifferentiation and ADM. When damage is resolved, inhibition of Kras signalling is necessary for promoting acinar re-differentiation and tissue regeneration. (B) Mutant Kras induces Gata4-dependent expression of genes that activate macrophages to trigger inflammation, and promotes cell survival and proliferation, which altogether results in acinar dedifferentiation and ADM. In this case, mutant Kras does not allow acinar re-differentiation. Permanent proliferative signals from active Kras* and inflammatory cytokines increase oncogenic stress and activate Gata4 to induce PanIN formation, therefore limiting the tumourigenic process.

TLR-mediated inflammation may be interpreted as a response to infection or tissue injury (the actual activators of TLRs). In such situations, TLR-expressing cells, which are trained to be the guardians of a tissue with high damaging potential, receive signals to avoid a catastrophe: the uncontrolledly release of digestive enzymes. To do so, they orchestrate an inflammatory response in order to promote tissue regeneration; they isolate acini to protect them from enzymes released into the interstitium (fibrogenic response); and they induce cells suffering from oncogenic stress to undergo a senescent program (PanIN development). In this context, it is conceivable that loss of the regulator Myd88 disrupts the mechanisms controlling inflammation, fibrogenesis and PanIN formation, resulting in enhanced tumourigenesis.

Another example of the tumour-limiting effect of PanINs and their surrounding microenvironment has been highlighted by the study of the fibrogenic response. Although to a lesser extent compared to PDAC, PanINs also develop a stromal response that results in deposit of extracellular matrix surrounding the lesion, a process mediated by activated PSCs that interplay with pro-inflammatory macrophages and DCs (Shi *et al*., 2014). Initially, fibrosis was considered as a barrier for drug delivery (Olive *et al*., 2009) and, therefore, its depletion was a promising strategy to improve patient outcome. However, it has been recently shown that the fibrogenic response has anti-tumoural effects as they limit cancer cells potential to be invasive (Özdemir *et al*., 2015; Rhim *et al*., 2014).

In line with these studies, our work also suggests a tumour-suppressing effect of PanINs. In the experiments where we show that PDAC develops in the absence of PanINs (*Kras**;*Gata4*$^{P-/-}$ mice) (Fig. 7B, 8B, 19A, 19B), we have consistently observed that these tumours present higher grading than the ones formed in *Kras** mice. In addition, we have provided with data showing that mice bearing Gata4-null tumours present poorer survival in a *Trp53*$^{-/-}$ background. Altogether, these results suggest that PanIN can limit tumour progression.

9. How does PDAC develop in *Kras**;*Gata4*$^{-/-}$ pancreas

Given that PDAC can develop in the absence of PanINs, we aimed to investigate the putative precursor that may progress to frank malignancy. A detailed analysis of *Kras**;*Gata4*$^{-/-}$ pancreata identified acinar cell clusters characterized by paleness, reduced eosinophilia, and a variable degree of hyperplasia, with or without enlarged lumina (ductal and acinar lesions, respectively), as the only abnormalities present in the pancreatic parenchyma. We designated these lesions "PALes" for "Pale Acinar Lesions", and we hypothesize that PALes are clusters of acinar cells initiating a Kras*-induced metaplastic process, which would progress into ADM/PanIN in a *Gata4*$^{+/+}$ context but not in a *Gata4*$^{-/-}$ genetic background (Fig. D6A). Indeed, other studies have inadvertedly reported

the presence of acinar PALes in *Kras*;Gata4*$^{+/+}$ mice (Fig. D6B) (Guerra *et al.*, 2011, supplementary data).

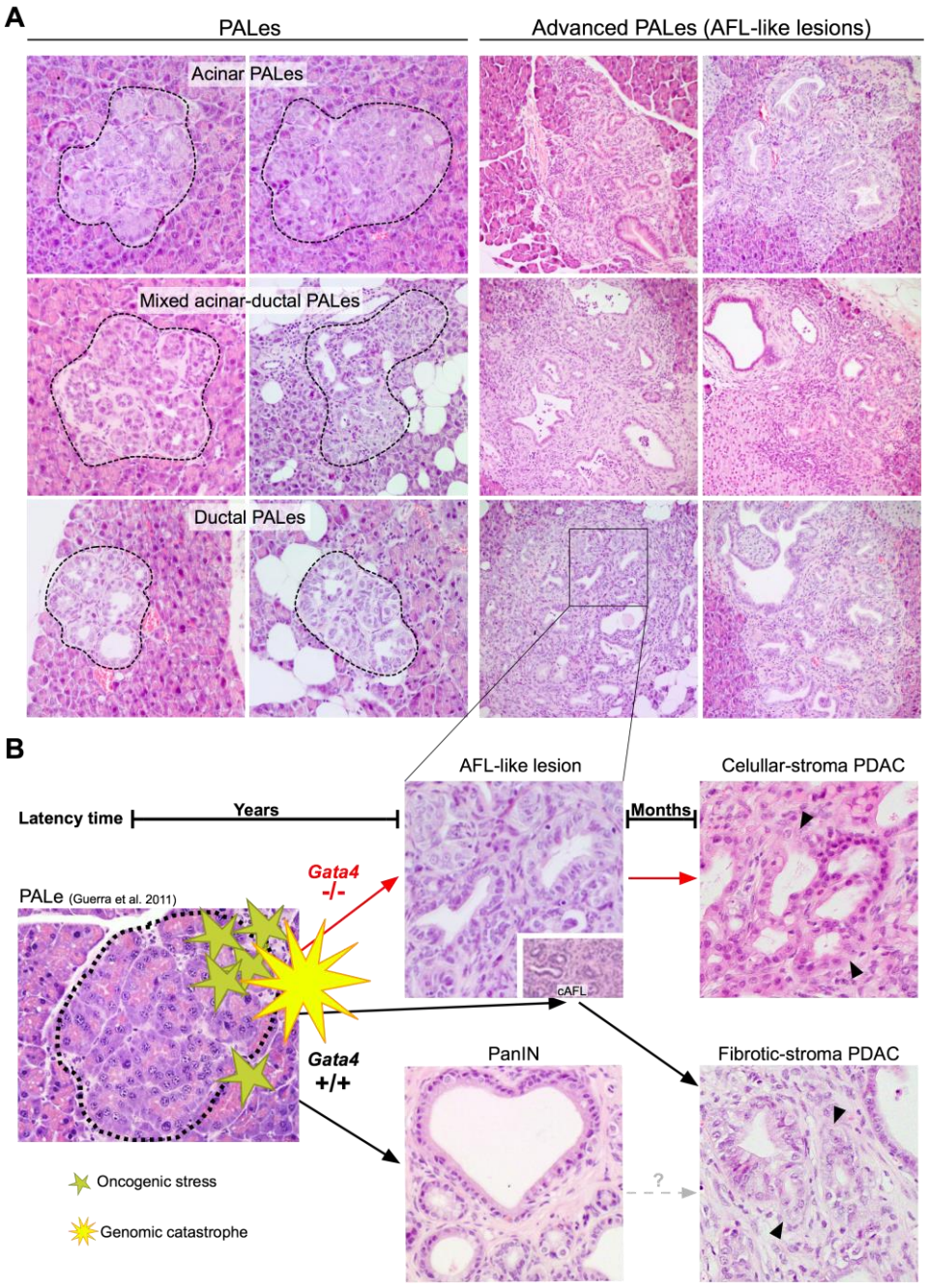

Figure D6. Hypothetical model of *Gata4*$^{-/-}$ PDAC development from AFL-like PAle lesions. **(A)** Representative images showing the different morphologies of small PALes, which are found in *Kras*;Gata4*$^{-/-}$ mice of all ages (left), and advanced PALes found only in *Kras*;Gata4*$^{-/-}$ mice older than 40w (right). **(B)** Proposed model of pancreatic carcinogenesis in *Kras** and

(Fig. D6 cont.) *Kras*;Gata4*$^{P-/-}$ mice. Oncogenic signalling derived from permanent Kras activation of pancreatitis activates tumour-suppressor mechanisms such as Gata4-mediated PanIN development. Accumulation of oncogenic stress results in a genomic catastrophe that inactivates tumour suppressors and result in development of PDAC precursors: classical AFL (cAFL) in Kras* mice and AFL-like lesions in *Kras*;Gata4*$^{P-/-}$. Although there is a long latency time before the genomic catastrophe occurs, AFL and AFL-like lesions rapidly progress to PDAC, which display a highly cellular or a fibrotic stroma in *Gata4*$^{-/-}$ and *Gata4*$^{+/+}$ PDAC, respectively. Arrowheads indicate infiltration foci, indicative of malignancy.

PALes characterized throughout this study could be frequently identified in mice of all ages, but advanced PALes (larger than PALes with increased atypia, but without reaching a PDAC status; earlier referred as AFL-like lesions) were much more scarce (Fig. D9A). A thorough quantification of these lesions is currently being performed, but a preliminary analysis shows that the incidence of advanced PALes resembles much more the incidence of tumours than that of early PALes. These results suggest that many PALes do not progress, but once they do, they rapidly develop into PDAC.

As commented earlier, we suggest that advanced PALes are AFLs that look different from the ones originally described (Aichler *et al*., 2012), as they present a low-fiber and highly cellular stroma, probably due to the lack of PSCs activation in a Gata4-null context. Indeed, a preliminary analysis of the PDAC stroma in the presence or absence of Gata4 reveals higher fibrogenic response in the first case and higher cellular content in the second (represented in Fig. 9B). Considering that the stromal response limits pancreatic tumourigenesis, these results may account for the recurrent observation that *Gata4*$^{-/-}$ PDACs are of higher tumour grade and yield a poorer survival in a p53-null context. If that was the case, the work presented here would strengthen the recent hypothesis pointing to AFL as true PDAC precursors (Aichler *et al*., 2012), while low-grade PanINs would represent mainly dead-end lesions activating a senescence program (Fig. 9B) (Caldwell *et al*., 2012, Guerra *et al*., 2011, Collado *et al*., 2005). These findings would be in agreement with the high frequency of low-grade PanIN in the normal population, which do not progress to PDAC (Matsuda *et al*., 2017). In this regard, it is noteworthy that PALE-like lesions have recently been identified in human pancreata (Hruban R, personal communication).

The proposed hypothesis is in agreement with a recent study showing that PDAC usually carries complex genomic rearrangements that are likely to be the cause of malignancy (Notta *et al*., 2016). This study proposes that PDAC does not necessarily develop through a particular sequence of genetic alterations (*KRAS* followed by *CDKN2A*, then *TP53* and *SMAD4*) and that the evolutionary trajectory of pancreatic cancer progression is not gradual and can be attributted to a single genomic rearrangement event that inactivate *en bloc* the tumour suppressors *CDKN2A*, *TP53* and *SMAD4.* These notions are consistent with the observation that 80% of pancreatic cancer patients present with advanced disease at diagnosis. The reasons for the occurrence of such catastrophic mitotic

phenomena are far from being understood, but their contribution to frank malignancy has been postulated (Rode *et al*., 2016).

10. GATA4 in human tumours

Our exploratory analysis of the role of GATA4 in human tumours has unveiled that GATA4 expression is absent from normal ducts but it is detectable in PanINs and in a fraction of patient's PDAC.

Given our previous findings of loss of GATA6 expression in basal-like tumours, we analysed GATA4 expression in the context of the molecular taxonomy of PDAC (P. Bailey *et al*., 2016; Collisson *et al*., 2011; Moffitt *et al*., 2015; Noll *et al*., 2016). GATA4 expression was also lower in tumours with a basal-like/squamous phenotype and low GATA4 expression is related to poorer survival. Interestingly, the combined analysis of GATA4 and GATA6 expression showed that patients with tumours having a low expression of both genes had the lowest survival, suggesting partially non-overlapping function for the two genes. Tumours expressing high-GATA4 also showed higher activity of the pathway "digestion", which includes several digestive enzymes, and therefore is indicative of cell differentiation. Contrarily, tumours expressing low GATA4 display higher activity of the pathways involved in cell cycle progression and mitosis. Altogether, these results suggest that GATA4 also plays a tumour suppressive role in human carcinogenesis, and that detection of GATA proteins in PDAC biopsies could be informative of patient survival.

11. Final remarks

Our work provides evidence that Gata4 is an important regulator of Kras*-induced inflammation and metaplasia. Unlike Gata6, Gata4 is not required to maintain acinar function, yet we suggest that Gata4 regulates an epithelial-hematopoitic signalling in the pancreas in the steady state. This regulation is not evident in basal conditions, but it becomes overt in a Kras* context, where the oncogene fails to induce inflammation and ADM/PanIN in the absence of Gata4. We propose that upon Kras* activation, Gata4 regulates the expression of factors that stimulate macrophages to elicit an inflammatory response, as well as it allows epithelial cells suffering from oncogenic stress to undergo PanIN transdifferentiation, whose senescent state limits carcinogenesis. Accordingly, Gata4-null PDAC presents higher grading and poorer survival in a *Trp53*$^{-/-}$ background. Overall, our results show that Gata4 has unique functions in the pancreas that are fundamental for developing a proper response to Kras*-induced oncogenic stress.

CONCLUSIONS

1. Gata4 is dispensable for pancreas development after E10.5 and, unlike Gata6, it is not required for the maintenance of acinar homeostasis in the adult.

2. *Gata4*$^{P-/-}$ mice develop tumours when mutant Kras expression is activated either in pancreatic progenitors or in adult acinar cells. However, they do not develop PanIN or acino-ductal metaplasia.

3. Gata4-null acinar cells can undergo metaplasia upon IL17 treatment *in vitro*, but fail to form PanINs upon IL17 administration *in vivo*, indicating that Gata4 is necessary for PanIN development.

4. Gata4 is required for the inflammatory response induced by mutant Kras in the pancreas.

5. Gata4 regulates the expression of soluble factors, including Reg3b, which activate macrophages to enhance a further inflammatory response.

6. In mice, *Gata4* behaves as a tumour suppressor gene when its inactivation is combined with that of *Trp53* and the activation of mutant *Kras*.

7. GATA4 is lost in a subset of human PDAC, especially in the squamous subtype. Patients with tumours showing low levels of expression of GATA4 and GATA have the poorest survival.

CONCLUSIONES

1. Gata4 es prescindible para el desarrollo del páncreas posteriormente a E10.5 y, contrariamente a Gata6, no se requiere para el mantenimiento de la homeostasis acinar en el adulto.

2. Los ratones *Gata4*$^{P-/-}$ desarrollan tumoures cuando la expresión de Kras mutante se activa tanto en progenitores pancreáticos como en células acinares adultas. Aun así, no desarrollan PanINs ni metaplasia acino-ductal.

3. Las células acinares deficientes en Gata4 pueden desarrollar metaplasia ante el tratamiento con IL17 *in vitro*, pero no son capaces de formar PanINs ante la administración de IL17 *in vivo*, indicando que Gata4 es necesario para el desarrollo de PanINs.

4. Gata4 se require durante la respuesta inflamatoria inducida por Kras mutante en el páncreas.

5. Gata4 regula la expresión de factores solubles, incluyendo Reg3b, que activan a macrófagos para aumentar una posterior respuesta inflamatoria.

6. En ratones, Gata4 se comporta como un gen supresor de tumoures cuando su inactivación se combina con la de *Trp53* y la activación de *Kras* mutante.

7. La expresión de GATA4 se pierde en un grupo de PDAC en humanos, especialmente en el subtipo escamoso. Los pacientes con tumoures que presentan niveles bajos de expresión de GATA4 y GATA6 tienen el peor pronóstico.

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